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CONTENTS

No. 1. February, 1946

PAGE

Kozloff, Eugene N.
Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). I. Hypocomina tegularum sp. nov. and Crebricoma gen. nov. 1
Short, Robert B.
Observations on the giant amoeba, Amoeba carolinensis (Wilson, 1900) 8
Schrader, Franz
The elimination of chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas 19
Hall, C. E., M. A. Jakus, and F. O. Schmitt
An investigation of cross striations and myosin filaments in muscle 32
Smith, F. G. Walton
Effect of water currents upon the attachment and growth of barnacles 51
D'Angelo, Ethel Glancy
Micrurgical studies on Chironomus salivary gland chromosomes 71
Serial Publications Added to the Marine Biological Laboratory and the Woods Hole Oceanographic Institute Library since February, 1943 88

No. 2. April, 1946

Costello, Donald Paul
The swimming of Leptosynapta 93
Richards, A. Glenn and Laurence K. Cutkomp
Correlation between the possession of a chitinous cuticle and sensitivity to DDT 97
Carlson, J. Gordon
Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis 109
Miller, Milton A.
Toxic effects of copper on attachment and growth of Bugula neritina 122
Walford, Lionel A.
New graphic method of describing the growth of animals 141
Bodenstein, Dietrich
Investigation on the locus of action of DDT in flies (Drosophila) 148
Hopkins, Dwight L.
The contractile vacuole and the adjustment to changing concentration in fresh water amoebae 158

60067

No. 3. June, 1946

Hayashi, Teru
Dilution medium and survival of the spermatozoa of Arbacia punctulata. II. Effect of the medium on respiration 177
Henley, Catherine
The effects of lithium chloride on the fertilized eggs of Nereis limbata 188
Kozloff, Eugene N.
Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). II. Hypocomides mytili Chatton and Lwoff, Hypocomides botulae sp. nov., Hypocomides parva sp. nov., Hypocomides kelliae sp. nov., and Insignicoma venusta gen. nov., sp. nov. 200
Tyler, Albert
Natural heteroagglutinins in the body-fluids and seminal fluids of various invertebrates 213
Krugelis, Edith Judith
Distribution and properties of intracellular alkaline phosphatases 220
Williams, Carroll M.
Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, Platysamia cecropia 234
Loosanoff, Victor L. and Charles A. Nomejko
Feeding of oysters in relation to tidal stages and to periods of light and darkness 244
Schrader, Franz
Autosomal elimination and preferential segregation in the harlequin lobe of certain Discocephalini (Hemiptera) 265
Rugh, Roberts
The effect of the adult anterior pituitary hormone on the tadpoles and the immature male frogs of the bullfrog, Rana catesbiana 291

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THE

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE CHATTON AND LWOFF (ORDER HOLOTRICHA, SUBORDER THIGMOTRICHA)

I. HYPOCOMINA TEGULARUM SP. NOV. AND CREBRICOMA GEN. NOV.

EUGENE N. KOZLOFF

Lewis and Clark College, Portland, Oregon

INTRODUCTION

The genus *Hypocomina* was proposed by Chatton and Lwoff (1924) to include a single species, *Hypocoma patellarum* Lichtenstein (1921), parasitic on the pallial branchiae of *Patella caerulea* L. Subsequently, Raabe (1934) described as *Hypocomina carinata* a ciliate from *Mytilus edulis* L. which Jarocki (1935) pointed out obviously does not belong to the genus *Hypocomina*. Raabe used the name *Hypocomina carinata* again in 1938, however, and no literature has come to my attention in which the form in question is assigned to another genus. In the present paper I will describe as *Hypocomina tegularum* sp. nov. an ancistrocomid ciliate from the ctenidium of *Tegula brunnea* (Philippi); and on the basis of my studies on the morphology of a ciliate which I presume to be identical with "*Hypocomina*" *carinata*, I will establish the position of Raabe's species in a new genus, *Crebricoma*.

HYPOCOMINA TEGULARUM SP. NOV.

(Plate I, Figs. 4, 5)

The body is elongated and compressed dorso-ventrally. Fifteen living individuals taken at random ranged in length from 26 μ to 36 μ, in width from 12 μ to 17 μ, and in thickness from 9 μ to 12 μ, averaging about 31 μ by 15 μ by 11 μ. As seen in dorsal view the curvature of the left margin of the ciliate is a little more pronounced than that of the right margin, although this asymmetry is not conspicuous and in most fixed specimens is barely apparent. The body is widest near the middle and is rounded posteriorly. The anterior end is attenuated, bent ventrally, and truncate at the tip. The reduced ciliary system, to be described presently, is disposed in a shallow, relatively flat depression occupying the anterior half of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the thigmotactic field are convex.

A contractile suctorial tentacle enables the ciliate to attach itself to the epithelial cells of the ctenidium of the host and to suck out their contents. I have not yet succeeded in determining the exact nature of the tentacle in the ancistrocomid ciliates which I have examined and the accounts of other authors regarding its structure are likewise unsatisfactory. In *Hypocomina tegularum*, it appears to be a membranous tube-like extension of the attenuated anterior end which is protracted and contracted at will. When fully extended the tentacle is about 3 μ in length and 1–1.5 μ in diameter. It is hoped that further studies on ancistrocomid ciliates will shed some light on the problem of the structure of this interesting organelle.

The internal tubular canal continuous with the suctorial tentacle is difficult to study in the living ciliates, but in material stained with iron hematoxylin or alum hematoxylin it can usually be traced posteriorly for a distance equal to about one-third the length of the body (Plate I, fig. 4, c). In most individuals it is directed obliquely to the right. In fixed specimens the extreme anterior portion of the canal is approximately 1–1.5 μ in diameter, while the remainder of it appears to be swollen and to have the form of a poorly defined clear area. Lichtenstein (1921) interpreted the comparable swelling of the canal in *Hypocomina patellarum* to be a "balle alimentaire." Jarocki (1935) attributed the clear area described by Raabe (1934) behind the tentacle of *Hypocomella macomae* and "*Hypocomina*" *carinata* to hyperdistension of the canal due to unequal infiltration by the fixing reagent or as a result of its action upon moribund, partly plasmolyzed individuals. It is interesting to note in this connection, however, that some ancistrocomid ciliates rarely show this phenomenon upon fixation and the canal can be traced to within a short distance of the posterior end of the body.

The delicate thigmotactic cilia of *Hypocomina tegularum* are about 6–7 μ in length and are disposed in nine longitudinal rows. All the rows originate about 3 μ or 4 μ posterior to the base of the suctorial tentacle and are about one-half the length of the body (Plate I, fig. 4). The first five rows from the right side of the ciliate are appreciably longer and sometimes appear to lie closer together than the other four rows. Chatton and Lwoff (1924) reported that in *Hypocomina patellarum* a ridge-like eminence (carène) divides the ciliary area into two unequal fields. These authors did not, however, state how many of the ten ciliary rows described by Lichtenstein (1921) for *H. patellarum* are in each complex and did not give any facts concerning the relative lengths of the rows comprising the two fields. In *Hypocomina tegularum* I have not discerned an unmistakable eminence separating the five rows on the right from the four rows on the left, although in some living and fixed individuals the distance between the distal portions of the fifth and sixth rows is somewhat greater than the distance between the other rows.

EXPLANATION OF PLATE I

FIGURE 1. *Crebricoma carinata* (Raabe) comb. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. × 1130.

FIGURE 2. *Crebricoma carinata* (Raabe) comb. nov. Dorsal aspect, from life.

FIGURE 3. *Crebricoma carinata* (Raabe) comb. nov. Lateral aspect from right side, from life.

FIGURE 4. *Hypocomina tegularum* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. × 1870.

FIGURE 5. *Hypocomina tegularum* sp. nov. Lateral aspect from left side, from life.

c = internal tubular canal, cv = contractile vacuole, fv = food vacuole, ma = macronucleus, mi = micronucleus.

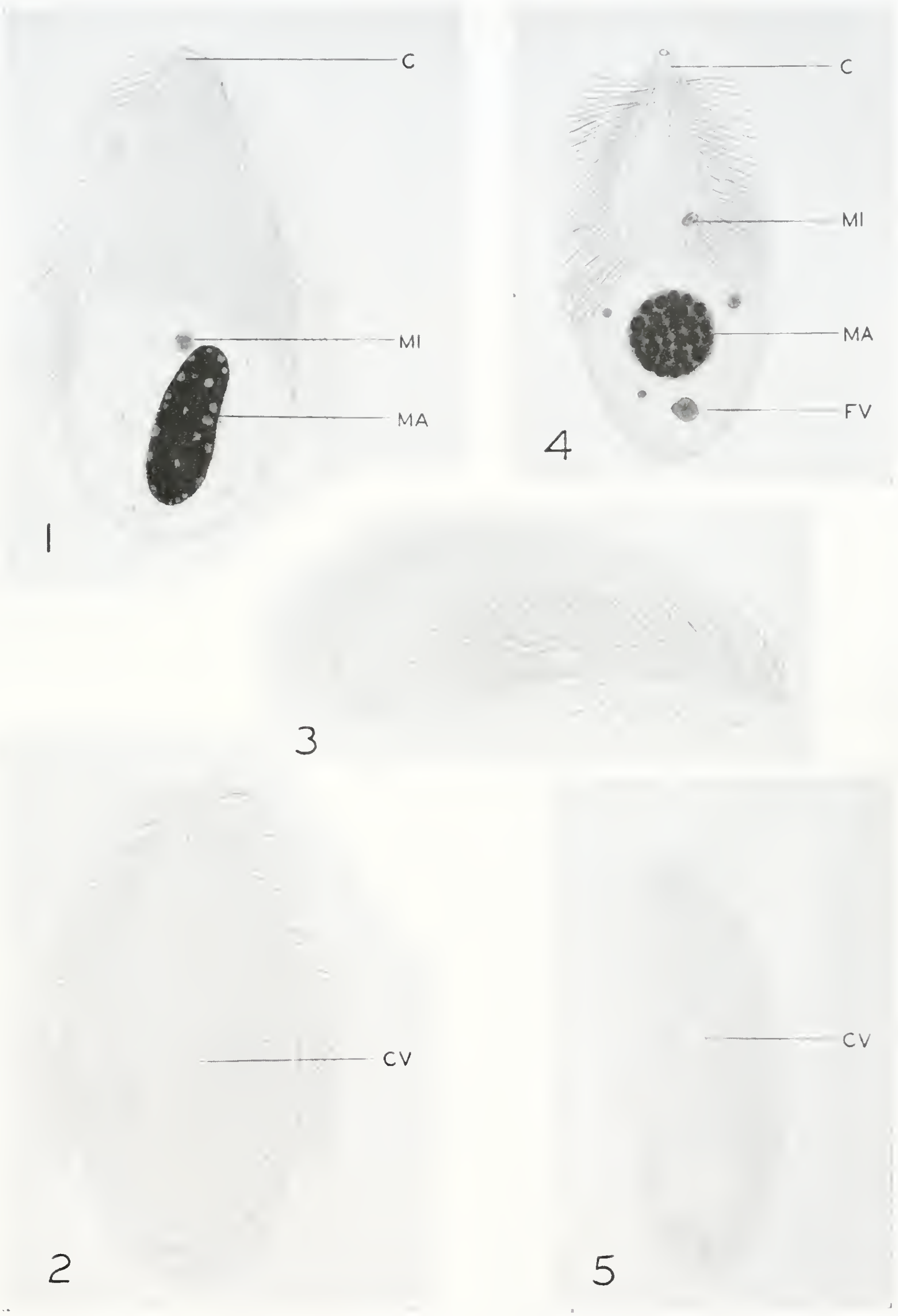
C
MI
MA
1
C
MI
MA
FV
4
3
CV
2
CV
5

Plate I

The cytoplasm is colorless and contains numerous food inclusions and refractile granules. The refractile granules are apparently lipoid droplets, as they are dissolved out by toluol used for clearing following staining. One or more large food vacuoles (Plate I, fig. 4, fv) are usually present in the posterior part of the body. The contractile vacuole (Plate I, fig. 5, cv) lies near the middle of the body and opens to the exterior on the ciliated ventral surface. I have not detected a permanent opening in the pellicle.

The spherical macronucleus (Plate I, fig. 4, ma) is situated in the posterior half of the body. In preparations stained with iron hematoxylin or alum hematoxylin the chromatin appears to be aggregated into many large, round granules. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the diameter of the macronucleus ranged from 5.5 μ to 7 μ.

The spherical or ovoid micronucleus (Plate I, fig. 4, mi) is usually situated anterior to the middle of the body. It is very difficult to detect in living individuals. In stained preparations the chromatin is dispersed into granules of varying size and shape lying for the most part near the periphery. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the diameter of the micronucleus ranged from 1.6 μ to 2.2 μ.

I found *Hypocomina tegularum* to be present in small numbers on the ctenidium of two of three specimens of *Tegula brunnea* (Philippi) which I collected at Carmel Bay, California, in September, 1944.[1] Since that time I have had the opportunity to examine a large number of individuals of *Tegula brunnea* from localities near Moss Beach and Dillon Beach, California, but found none to be infested by *Hypocomina tegularum.*

Hypocomina tegularum sp. nov.

Diagnosis: Length 26 μ–36 μ, average about 31 μ; width 12 μ–17 μ, average about 15 μ; thickness 9 μ–12 μ, average about 11 μ. The anterior end is attenuated, bent ventrally, and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary rows are nine in number and are disposed in a shallow depression occupying the anterior half of the ventral surface. The first five rows from the right are slightly longer than the other four rows. The macronucleus is spherical and is situated in the posterior half of the body. The micronucleus lies anterior to the middle of the body. Ectoparasitic on the ctenidium of *Tegula brunnea* (Philippi) (Carmel Bay, California). Syntypes are in the collection of the author.

CREBRICOMA GEN. NOV.

CREBRICOMA CARINATA (RAABE) COMB. NOV.

(Figure 1. Plate 1, Figs. 1–3)

I have distinguished two species of ancistrocomid ciliates parasitic on the epithelium of the palps and ctenidial filaments of *Mytilus edulis* L. from various localities in San Francisco Bay. One of these is *Hypocomides mytili* Chatton and Lwoff; the other I presume to be identical with "*Hypocomina*" *carinata* Raabe, for which I propose a new genus, *Crebricoma.* My observations on the morphology of this species permit me to augment the original description given by Raabe. It seems advisable, therefore, to present herein a revised description of *Crebricoma carinata*.

---

[1] I am indebted to Dr. D. T. MacDougal for his kindness in arranging my trip to Carmel which made possible the collection of the original material of the ciliate described herein.

The body is elongated and somewhat compressed dorso-ventrally. Fifteen living individuals taken at random ranged in length from 58 μ to 71 μ, in width from 27 μ to 39 μ, and in thickness from 22 μ to 31 μ, averaging about 64 μ by 31 μ by 25 μ. The anterior end is narrowed, bent ventrally, and in dorsal view is seen to end in an oblique truncation, the right side of which projects a little more than the left side (Plate I, figs. 1, 2). The body is widest near the middle and is rounded posteriorly. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior two-thirds of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the thigmotactic area are convex.

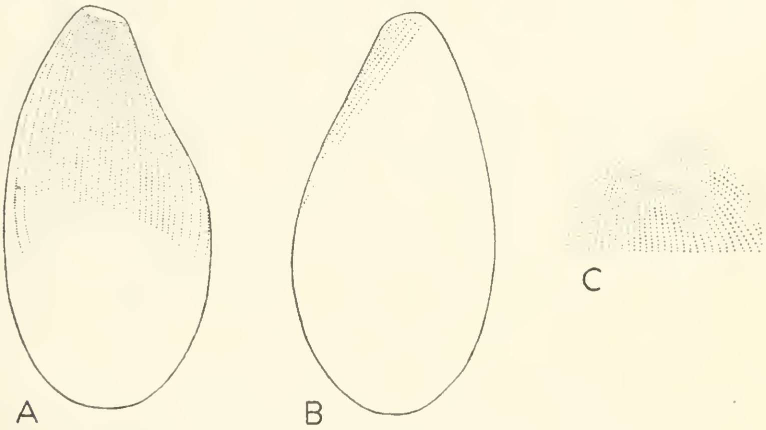

FIGURE 1. *Crebricoma carinata* (Raabe) comb. nov. Distribution of ciliary rows. Semi-diagrammatic representations based on camera lucida drawings of specimens fixed in Schaudinn's fluid and impregnated with activated silver albumose (protargol).

A. Ventral aspect, B. Dorsal aspect, C. View of anterior end.

The suctorial tentacle is situated on the right side of the anterior truncation. I am at present unable to make any conclusive statement with regard to the structure and contractile properties of the tentacle in this species. The internal tubular canal continuous with the tentacle cannot be satisfactorily distinguished in living material, but can be demonstrated in some fixed specimens which have been stained with iron hematoxylin. It appears to pass at first dorsally and then ventrally and obliquely to the right. I have not succeeded in tracing it posteriorly for more than a short distance. A broad lighter area behind the anterior part of the canal is frequently observed in fixed individuals.

As described by Raabe, the ciliary system of *Crebricoma carinata* consists of about twenty closely-set rows bordered on the right by two longer and more widely-spaced rows and on the left by three such rows. According to my observations, however, the more widely-spaced rows on the left side do not represent a complex separate from the closely-set rows. The number of rows in the thigmotactic field of *C. carinata* is very difficult to determine, since some of the rows on the left are lateral in position and several of them originate on the dorsal surface. In one individual impregnated with activated silver albumose I counted thirty-four rows and in another individual, thirty-six rows. In all specimens which I have examined carefully the number of ciliary rows exceeded thirty-two.

The two widely-spaced rows on the right side of the thigmotactic field originate on the ventral surface near the base of the suctorial tentacle. The outer row is the longer and is about two-thirds the length of the body (Fig. 1A). All the rows of the second complex, with the exception of three or four rows on the extreme left which are about as widely-spaced as the two long rows on the right side, are very closely-set and are one-half to two-thirds the length of the body, becoming progressively longer toward the left. Several of the rows on the left side of the thigmotactic field originate on the left lateral margin or on the dorsal surface (Fig. 1B; Plate I, fig. 2). The arrangement of the ciliary rows at the anterior end thus forms an incomplete suture (Fig. 1C) reminiscent of the anterior field of other holotrichous ciliates.

The cilia of *Crebricoma carinata* are about 10–11 μ in length. When the organism is attached to the epithelium of the palps or ctenidial filaments of the host the cilia usually exhibit only a sluggish movement. Occasionally one or two small groups of cilia near the anterior end beat energetically. When dissociated from the host *C. carinata* swims actively and its cilia beat metachronously.

Raabe proposed the specific name *carinata* in allusion to a keel-like prominence on the dorsal surface. I have seen such prominences on seriously shrunken fixed specimens of *Crebricoma carinata* and on some living individuals which were obviously undergoing plasmolysis. I have not, however, been able to distinguish them on ciliates freshly removed from the host. Raabe also described a sunken space between the keel-like prominence and the outermost ciliary row on the right side, which he said "vermutlich dem von Chatton und Lwoff bei anderen Hypocomiden beschriebenen 'vestige de frange adorale' entspricht." I do not believe such a depression exists in normal individuals, although I have observed groove-like and slit-like depressions form on the lateral and dorsal surfaces of moribund specimens of *Crebricoma carinata,* as well as *Hypocomides mytili.*

The cytoplasm is colorless and contains a few small refractile granules, which presumably are lipoid droplets, and food inclusions. I have not discerned any large food vacuoles in this species. The contractile vacuole (Plate I, fig. 2, cv) opens to the exterior on the ventral surface near the middle of the body. There appears to be no permanent opening in the pellicle.

The sausage-shaped or ovoid macronucleus (Plate I, fig. 1, ma) is situated in the posterior half of the body. In preparations stained with iron hematoxylin or the Feulgen nuclear reaction the chromatin is aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. Viewed dorsally, the longitudinal axis of the macronucleus is ordinarily placed obliquely to the longitudinal axis of the body. In most specimens the anterior end of the macronucleus is directed dorsally, while the posterior end is directed ventrally. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction the macronucleus ranged in length from 13 μ to 24.3 μ and in width from 5.6 μ to 11.7 μ.

The spherical micronucleus (Plate I, fig. 1, mi) is readily detected in the living ciliate. It is commonly situated near the dorsal surface close to the anterior end of the macronucleus. Following fixation the micronucleus stains lightly with iron hematoxylin and the Feulgen reaction. The chromatin appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.7 μ to 3.6 μ.

Crebricoma gen. nov.

Diagnosis: The body is elongated, somewhat flattened dorso-ventrally, and narrowed anteriorly. The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary rows are numerous and except for some which originate on the dorsal surface or left lateral margin are disposed on the ventral surface. Two long, widely-spaced rows on the right side of the ciliary system appear to form a complex separate from the remaining rows, which are for the most part closely-set. The arrangement of the ciliary rows at the anterior end of the body forms an incomplete suture. Genotype: *Crebricoma carinata* (Raabe) comb. nov. (= *Hypocomina carinata* Raabe).

Crebricoma carinata (Raabe) comb. nov.

Diagnosis: Length 58 μ–71 μ, average about 64 μ; width 27 μ–39 μ, average about 31 μ; thickness 22 μ–31 μ, average about 25 μ. Two widely-spaced rows of cilia on the right side, the outer of which is about two-thirds the length of the body, and a series of more than thirty rows, which with the exception of three or four on the left are closely-set, comprise the ciliary system. The closely-set rows are one-half to two-thirds the length of the body, becoming progressively longer toward the left. The macronucleus is sausage-shaped or ovoid. The micronucleus is spherical. Ectoparasitic on the palps and branchial filaments of *Mytilus edulis* L.

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OBSERVATIONS ON THE GIANT AMOEBA, AMOEBA CAROLINENSIS (WILSON, 1900)[1]

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INTRODUCTION

During the past few years there has been considerable controversy over the name of the giant fresh-water amoeba which Wilson (1900) described and named *Pelomyxa carolinensis*. He placed this amoeba in the genus *Pelomyxa* evidently because it has many nuclei.

Mast (1938) and Rice (1945) agree with Wilson in regard to the name of this form. The latter author says that the principal characteristic of the genus *Pelomyxa* is its many nuclei. Greeff (1874), who established the genus, states that besides vacuoles there are in *Pelomyxa palustris* three kinds of characteristic structures: (1) Nuclei, (2) Hyaline and homogeneous bodies of spherical, ellipsoidal, or irregular shape, and (3) *Stäbchen,* little rods (later considered to be symbiotic bacteria). He does say that the large number of nuclei forms *a* principal characteristic of the genus *Pelomyxa*. However, since Greeff's description of *P. palustris,* pelomyxae have been described with one or few nuclei (*P. Belevskii* Penard, 1888; *P. binucleata* (Gruber, 1885) Penard, 1902; *P. paradoxa* Penard, 1902; *P. lentissima* Schaeffer, 1918; *P. schiedti* Schaeffer, 1918), and amoeboid forms other than pelomyxae have been described with more than one nucleus. *Amoeba proteus* has been observed in this laboratory with as many as eight nuclei.[2] Also the hyaline spherical bodies are not found exclusively in *Pelomyxa*. In view of these facts, Penard (1902) states that the chief criterion for the genus *Pelomyxa* is the presence of symbiotic bacteria in the cytoplasm. He says, "S'il me fallait donc caractériser le genre Pelomyxa, je le ferais à peu près en ces terms: 'Amibes à mouvements lents, toujours pourvues de bactéries symbiotiques.'" He then points out that Wilson's rhizopod has in common with the *Pelomyxa* only the *Glanzkörper* and that this form has nothing to distinguish it from the genus *Amoeba*.

Schaeffer (1918) in describing *Pelomyxa lentissima* states, "Other inclusions in the ectoplasm are the bacterial rods, distinctive of the genus." Later in his account of *P. schiedti* he says, "The bacterial rods, the presence of which characterizes the genus *Pelomyxa,* are found in considerable numbers in *schiedti*." Bourne (1891) in speaking of *P. viridis* describes the organism as, "densely packed with bacteria."

Thus it is seen that the chief characteristic of the genus *Pelomyxa* is the presence of symbiotic bacteria in the cytoplasm. Since the giant amoeba does not possess these bacteria, it is not a *Pelomyxa*.

---

[1] The author wishes to express his grateful appreciation to Dr. B. D. Reynolds for his suggestions and helpful advice in regard to this work and to thank Dr. C. M. Gilbert for help in taking the photomicrographs.

[2] Personal communication from Dr. B. D. Reynolds.

Schaeffer (1926, 1938a) maintains that Wilson's amoeba represents Roesel's (1755) "der kleine Proteus," and should therefore be called *Chaos chaos* Linnaeus. But Roesel's description is entirely inadequate to establish a species or even a genus. He undoubtedly had an amoeboid form, but it is impossible to find out the exact structure of his "Proteus." He mentions granules (*Körnern*) but says nothing of vacuoles, which in the giant amoeba are much larger than the crystals (granules). Schaeffer seems to base his conclusion mainly on the size and shape of the "Proteus." He contends that Roesel was a reliable investigator and that his figures may therefore be credited with general accuracy. As Schaeffer (1926) points out, "Roesel states the natural size of his amoeba in the rounded (spherical?) form to have been the same as figure A, which measures about 1660 μ in diameter." Yet Schaeffer (1938a) says, "*Chaos chaos* has a distinctive size range which fluctuates around 500 μ diameter." Thus, Schaeffer himself gives the diameter of the giant amoeba as about $\frac{1}{3}$ that of Roesel's "Proteus." There are also striking differences between Roesel's figures and description of a binary fission in his "Proteus" and Schaeffer's (1938b) photographs and account of the "3-daughter division of the giant amoeba."

Mast and Johnson (1931) review the data presented by Roesel and express the opinion that the latter possibly was dealing with a myxomycete. Rice (1945) states, "It is impossible to ascertain the exact structure of Roesel's 'der kleine Proteus.'" Thus since both the generic and specific names, *Chaos chaos*, are based on Roesel's inadequate description, they are not valid.

Hegner and Taliaferro (1924) in referring to Wilson's large amoeba use the name *Amoeba carolinensis* for it, though they give no reason for doing so. The present investigation adds evidence to support the use of this name. Therefore, *Amoeba carolinensis* (Wilson, 1900) Hegner and Taliaferro, 1924 is considered to be the correct name of the organism used in these experiments.

CYTOLOGY AND PHYSIOLOGY

Materials and Methods

The original stock culture of *Amoeba carolinensis* was procured from the General Biological Supply House, Chicago, Illinois, in November 1944. About every two weeks subcultures were made in the following manner: Spring water containing five wheat grains per 100 cc. of water was boiled for a few minutes and left uncovered for several days. One or two of the grains of wheat were put into a butter dish and about 90 cc. of the boiled water was poured in, giving a depth of $\frac{3}{4}$ inches. The amoebae along with food organisms, which consisted chiefly of rotifers, *Chilomonas paramecium*, *Colpidium colpoda*, and *Paramecium caudatum*, were then added. In the best cultures a water mold grew on the wheat grains and many amoebae often were found among the hyphae of the mold. These stock cultures had been maintained for a little more than four months when the experiments began.

In addition to the wheat culture medium (W. C. M.) a hay medium (H. C. M.) was made as follows: 10 grams of chopped timothy hay were added to 1000 cc. of spring water and boiled for about 15 minutes. The solution was filtered to remove the solid particles and then put into test tubes with cotton stoppers and autoclaved on two successive days at 10 pounds pressure. From these test tubes the medium was taken to start new cultures during the experiments. After trying various concentrations of this hay medium it was found that the amoebae and food organisms remained healthier and increased more rapidly if the stock solution was diluted with four parts of boiled and aerated spring water.

Cultures of *Chilomonas paramecium* and *Paramecium caudatum* were established in both the wheat and the hay culture media to serve as food organisms for the amoebae during the experiments. Subcultures were made from these about every two weeks.

Forty specimens of *Amoeba carolinensis* were washed six times in boiled spring water. Ten were transferred to two stender dishes (five to each dish) containing paramecia in W. C. M. In a similar manner ten were transferred to two stender dishes containing chilomonads in W. C. M.; ten, to two stender dishes containing paramecia in H. C. M.; and ten, to two stender dishes containing chilomonads in H. C. M. Each day the cultures were inspected and the supply of food organisms was replenished if it was low. The amoebae were counted daily for seven-day periods and then on the seventh day (in some cases a few days later) new cultures were made from the ones of the preceding week, so that by the end of the experiment most of the amoebae had been growing in one kind of medium and feeding on one kind of food organism for at least 46 days. With the exception of two cultures, the pH was taken on each culture on or after the seventh day. During the experiment, observations as to the relative size and form of the amoebae were made; some of the amoebae in each culture were examined in hanging drops under the compound microscope; and at the end of the experiment photomicrographs were taken of a few amoebae in the wheat-culture medium.

RESULTS

After the first two weeks there were marked differences in the rates of reproduction, especially between those amoebae grown in W. C. M. and fed on paramecia and the other cultures, as can be seen in Table I. During the first two weeks there seems to be a period of adjustment when the reproduction rates of the different cultures do not vary significantly. However, during the third to sixth weeks the amoebae grown in W. C. M. and fed on paramecia increased rapidly—from 10 to 45 during the sixth week. The other cultures showed little or no growth.

The hydrogen-ion concentrations remained rather constant. The pH's varied from 7.0 to 8.1, with that of the H. C. M. containing paramecia usually a little higher than the others. The room temperature varied from about 20° to 26° C.

There was no significant difference in size of the amoebae in the various culttures, but there was a difference in form. Those growing in W. C. M. and fed on paramecia were often somewhat disc-shaped with short, blunt pseudopods radiating from all sides as shown in Figure 1a. Others were usually monopodal or bipodal with blunt pseudopods as shown in Figure 1b. Clumping of the disc-shaped amoeba was observed five or six times.

The amoebae in W. C. M. fed on chilomonads were more flattened, with thinner pseudopods which were often at right angles to the main part of the body and frequently branched (Fig. 1c). The pseudopods often had little knob-like swellings at their distal ends (Fig. 1d). The amoebae in these cultures occasionally were monopodal in form.

TABLE I

Effect of culture media and food organisms on the reproduction rates of *Amoeba carolinensis*. At the beginning of each week amoebae were put into new cultures corresponding to the conditions under which they previously had been growing, so that the end results represent cumulative effects. Since there always were two cultures of each type, the pH's of both cultures are given.

	Wheat medium with paramecia				Wheat medium with chilomonads				Hay medium with paramecia				Hay medium with chilomonads			
	Number of amoebae		% increase	pH	Number of amoebae		% increase	pH	Number of amoebae		% increase	pH	Number of amoebae		% increase	pH
	First day	Seventh day			First day	Seventh day			First day	Sixth day			First day	Seventh day		
First week	10	17	70%	7.5 7.6	10	22	120%	7.5 7.5	10	18	80%	7.7 7.8	10	11	10%	7.5 7.5
										Seventh day						
Second week	10	15	50%	7.6 7.6	9	14	55½%	7.5 7.6	10	11	10%	8.0 7.9	10	10	0%	7.8 7.7
Third week	10	30	200%		10	10	0%	7.8 7.9	10	16	60%	8.0 8.1	10	10	0%	8.0 8.1
Fourth week	10	48	380%	7.0 7.1	10	10	0%	7.6 7.6	10	10	0%	7.6 7.4	10	11	10%	7.9 8.0
Fifth week	10	57	470%	7.5 7.4	10	13	30%	7.6 7.6	10	5	−50%	7.6 7.4	10	10	0%	7.6 7.6
Sixth week	10	45	350%	7.3 7.4	10	12	20%	7.4 7.4	5	4	−20%	7.8 7.6	10	10	0%	7.3 7.2

In H. C. M. the animals differed very little from each other as far as external appearance is concerned. They were, for the most part, monopodal or bipodal. Sometimes, however, those with the chilomonad diet exhibited the branched form characteristic of the amoebae in W. C. M. with chilomonads. Those amoebae with the paramecium diet were usually slightly larger than the ones feeding on chilomonads, and during the last two weeks one specimen grew to be the largest amoeba in any of the cultures. Very large vacuoles were apparent in the amoebae in H. C. M. with the paramecium diet, and some individuals were spherical. These last two characteristics are probably associated with degeneration.

In Wilson's description (1900) of this giant amoeba, he mentions minute, elongate, and fusiform crystals in the endoplasm. Wilber (1942) describes the crystals in more detail and says that there are two types: (1) plate-like crystals, and (2) bipyramidal crystals, which are the more numerous. He states that these crystals (presumably both kinds) are formed from food in the food vacuoles. Wilson also mentions spherical bodies 8 μ in diameter and smaller, which resemble oil drops. Wilber gives the size range of these bodies as 2.5–8 μ in diameter, and

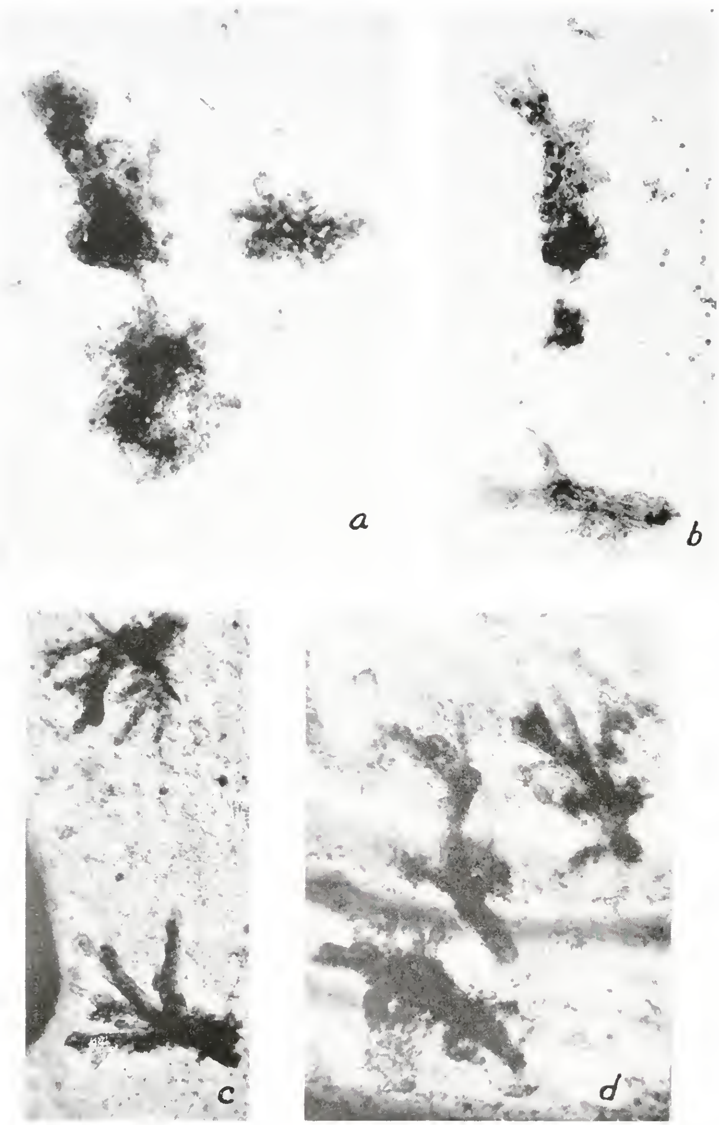

FIGURE 1. Photomicrographs of amoebae grown in wheat culture medium. a and b, those with paramecia as food organisms. c and d, those with chilomonads as food organisms.

concludes (1945) that they are formed from the crystals and the vacuole refractive bodies. He then says that the refractive spherical bodies function as reserve food in the amoeba.

While examining the stock and experimental amoebae under the compound microscope, differences in regard to the bipyramidal crystals and spherical bodies were noted. The plate-like crystals were so few in number that it was difficult to make a valid comparison in regard to their relative numbers and sizes.

In the stock cultures the amoebae had few spherical bodies, the largest of which were about 5 μ in diameter. The largest bipyramidal crystals were about 2.4 μ long.

The amoebae grown in W. C. M. with a diet of paramecia showed some larger bipyramidal crystals 2.8 μ long and a great number of smaller ones. There were few spherical bodies and the largest were only 3.1 μ in diameter.

Those specimens grown in W. C. M. and fed on chilomonads showed practically the same condition in regard to the bipyramidal crystals. However, there were very many large spherical bodies measuring 8.5 μ in diameter.

The spherical bodies of the amoebae grown in H. C. M. and fed paramecia were slightly more numerous and a little larger than the ones in the amoebae grown in W. C. M. with a paramecium diet. There were a few larger bipyramidal crystals 4.2 μ long, more 2.8 μ long and some smaller ones.

The amoebae grown in H. C. M. with chilomonads showed many large bipyramidal crystals 4.2 μ long, and the spherical bodies were very large and numerous. Many of the latter were 9.8 μ in diameter, and some were slightly flattened on one side so that they looked like a three-quarter-full moon.

Discussion

With the exception of size and number of nuclei, *Amoeba proteus* and *A. carolinensis* are very much alike. Schaeffer (1926) states, "My study of this matter (a comparison of *A. proteus* and *A. carolinensis*) leads me to include *diffluens* (*proteus*) in the same genus with *Chaos* (the giant amoeba). These two species, as a matter of fact, resemble each other more closely than most other species within one genus."

From Wilber's (1942) description of the cytology of *A. carolinensis* it is seen that the giant amoeba resembles *A. proteus* very closely in regard to the shape and structure of the nuclei and the cytoplasmic inclusions. Wilber (1945) has also shown that these two forms are very much alike in respect to the function of the nuclei, the formation of the contractile vacuoles, and the formation and function of the spherical bodies.

When the data of these experiments are compared with similar work on *A. proteus*, it is shown that these two amoebae resemble each other in nutritional requirements. The numbers and relative sizes of the crystals and spherical bodies in the amoebae in my experiments agree in general with the results of similar studies by Mast (1939) and Mast and Hahnert (1935) on *A. proteus*. Concerning the numerous spherical bodies in the chilomonad-fed amoebae, Mast and Hahnert say, "This is of considerable importance for, as stated above, it indicates that the neutral red staining droplets found in abundance in *Chilomonas* but not in *Colpidium* function in the formation of the spherical bodies in *Amoeba*." My experiments indicate that this is also the case in *A. carolinensis*.

In examining the data of these nutrition studies it is seen that in general the amoebae with the most numerous and largest crystals and spherical bodies had the lowest reproduction rates. In view of the fact that the spherical bodies serve as reserve food (Wilber, 1945), these experiments indicate that even though chilomonads may be ingested and digested by the amoebae, this diet is not adequate for normal reproduction. The reproduction rates in the different cultures agree with the results of Reynolds (1938) and Williamson (1944) for *A. carolinensis*, but do not correspond with Williamson's data on *A. proteus*.

The relative sizes of the amoebae in the various cultures do not agree very closely with the observations during similar experiments by Mast (1939) on *A. proteus* and Williamson (1944) on *A. proteus* and *A. carolinensis.* In my experiments there was no increase in the size of the amoebae which had eaten ciliates, as found by both these authors, nor did the chilomonad-fed amoebae decrease in size, as found by Williamson.

In regard to the form of *A. carolinensis* while utilizing paramecia as food organisms, the results are in accord with those of Mast and Root (1916) for *A. proteus* and Williamson (1944) for *A. proteus* and *A. carolinensis.*

NUCLEAR DIVISION

Materials and Methods

Specimens were taken from the stock cultures and fixed with Carnoy's, Belling's, and Bouin's fixatives. Those fixed with Carnoy's and Belling's fluids were stained with Delafield's and Heidenhain's haematoxylin. Those fixed in Bouin's fixative were stained with Heidenhain's haematoxylin. Some specimens were sectioned at 10 μ. Since those fixed with Carnoy's fixative and stained with Heidenhain's haematoxylin showed the nuclear structure best and stained the cytoplasmic inclusions very little, all the remaining work on nuclear divisions was done with these reagents.

In order to secure nuclear divisions, slides were made at various hours of the day. All the specimens showing nuclear division stages were fixed between 5:45 p.m. and 11:00 p.m. and were more or less spherical in form. The experiments are not extensive enough to warrant any statement as to a periodicity of mitosis. This seeming periodicity was probably a result of better selection toward the end of the experiment.

Observations and Descriptions

Resting nucleus

The resting nucleus of *A. carolinensis* (Fig. 2a) is disc-shaped, and measures about 24 × 10 μ. Immediately beneath a distinct nuclear membrane and adhering closely to it are darkly staining granules (peripheral granules), the largest of which are about 2 μ in diameter. The interior of the nucleus has a finely granular appearance and stains more lightly than the peripheral granules.

Schaeffer (1938) states that all the nuclei of an individual divide at the same time. Yet, nuclei at slightly different stages of division can be found in the same animal.

Prophase

In early prophase the nucleus enlarges apparently by a pulling away of the membrane from the central granules leaving a large endosome about 19 μ in greatest diameter. The nucleus now measures 27 × 15.4 μ. Most of the peripheral granules are still rather small and stain more darkly than the endosome. Some of them however are slightly larger and less deeply stained than those in the resting nucleus. Faint strands running from the endosome to the periphery are now apparent.

In a little later stage (Fig. 2b and c) the nucleus has become more spherical measuring about 27 × 22 μ. Some of the peripheral granules have become loosened

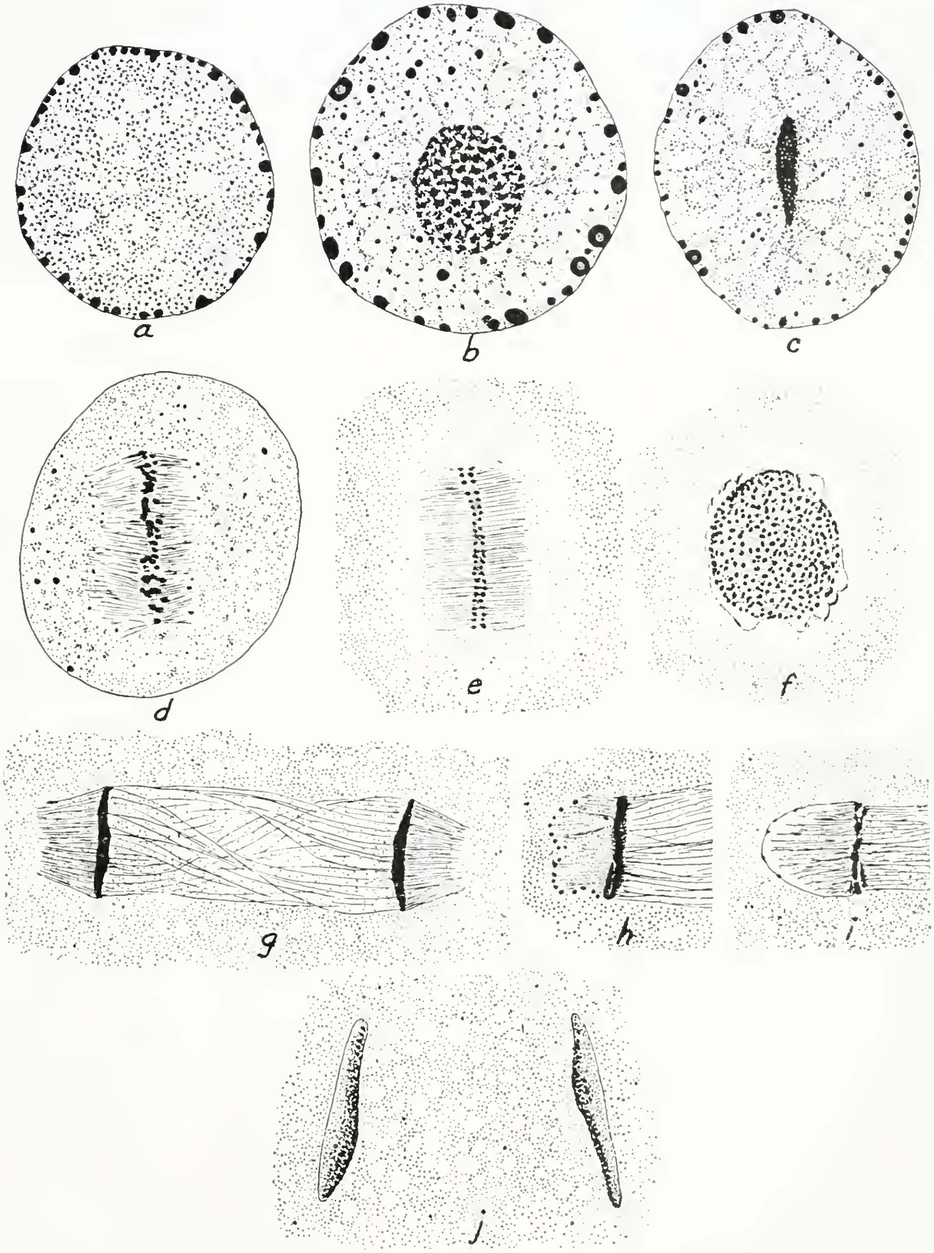

Figure 2. Camera lucida drawings of the stages of mitosis in *Amoeba carolinensis*. a. resting nucleus; b. prophase, face view; c. prophase, edge view; d. very late prophase showing chromosomes and spindle forming; e. metaphase, edge view; f. metaphase, polar view; g. late anaphase; h. early telophase, showing one plate with granules at the pole; i. a little later stage showing one pole with a delicate membrane and granules; j. late telophase with membrane fully formed. All drawings × 1660.

from the membrane, are more spherical, and show a lighter area in the center. The endosome has become smaller and more compact, and now stains about as deeply as the peripheral granules. At this stage the endosome is a thin disc measuring 9.3 μ in diameter and 1.5 μ in thickness. The reticulum connecting the endosome with the periphery is more evident now.

Very late prophase (Fig. 2d) shows the chromosomes becoming arranged on the plate which is 14.2 μ in diameter. Spindle fibers are distinct and the peripheral granules have practically disappeared. A lighter area in the cytoplasm immediately surrounding the nucleus is visible. The nucleus shown in Figure 2d was the only nucleus in the animal in this condition. The remaining nuclei were in metaphase or early anaphase.

Metaphase

At metaphase (Fig. 2e and f) the nuclear membrane has disappeared. There are, however, around the periphery of the plate delicate, blister-like structures which may be remnants of the nuclear membrane. The chromosomes, which are spherical or ellipsoidal, and perhaps about 300 in number, are arranged on a discoidal plate 13.2 μ in diameter. The split halves of the chromosomes in some cases can be seen. The spindle fibers are at right angles to the chromosome plate and are about 4 μ long. No centrioles or granules are apparent at the ends of the spindle fibers. There is still a lighter area in the cytoplasm around the figure.

Anaphase

During anaphase (Fig. 2g) the chromosome plate splits into two daughter plates which diminish in diameter with the chromosomes becoming so closely aggregated that they no longer can be distinguished individually. In late anaphase the plates measure 8.4 μ in diameter and are, for the most part, flat. A few are slightly arched or saucer-shaped. The spindle between the chromosome groups has in most cases become twisted as if both plates had rotated in opposite directions. The polar fibers seem to be finer and more numerous than the interzonal ones, and the polar areas appear slightly darker than the surrounding cytoplasm. The polar fibers have not shortened during anaphase but are in most cases 5 μ long, and the outside ones are inclined at an angle of about 60° to the plates.

In an amoeba containing thirty-three nuclei, twenty-nine are in late anaphase and four are in early telophase. The nuclei in anaphase have their polar fibers inclined at an angle of 60° to the chromosome plates. The four in early telophase have granules arranged on a more or less hemispherical surface or membrane (Fig. 2h and i). Twenty-nine of the spindles lie so that the distance between daughter chromosome groups can readily be measured. The average distance between groups is 34 μ. The shortest distance is 10 μ, and the longest distance is 62 μ. Some of the chromosome plates are tilted at angles to each other so that one of a pair is seen in edge view, while the other is seen in polar view. Some pairs of plates, both showing in edge view, are twisted at angles of 30° to 150° to each other. In the twenty-one anaphase spindles which lend themselves to analysis, twenty have the spindle between the chromosome groups twisted clockwise, or to the apparent right (Fig. 2g). One spindle shows no twisting. This constancy in direction indicates that the twisting is caused not by external forces in the cytoplasm, but by forces inherent in the spindle apparatus.

Telophase

In early telophase (Fig. 2h) granules appear at the distal ends of the polar fibers. A delicate, more or less hemispherical, membrane forms at the poles and the ends of the fibers nearest the poles disappear (Fig. 2i).

Later telophase (Fig. 2j) shows the plates larger in diameter, less dense and more finely granular, with the membrane more flattened. The granules are more densely packed on the median sides of the nuclei where the membrane is scarcely visible except near the edge of the disc. The spindle fibers have completely disappeared by this time.

DISCUSSION

Mitosis in *A. carolinensis* as herein described is very similar to that of *A. proteus* as described by Chalkley and Daniel (1933), Chalkley (1936), and Liesche (1938). The figures and descriptions of the nuclear division stages of *A. proteus* agree with those of *A. carolinensis* with the following exceptions:

1. The nuclei and mitotic figures of *A. carolinensis* are approximately half the size of those of *A. proteus*. Also the chromosome number of *A. carolinensis* (probably near 300) seems to be about half that given by Liesche (500–600) for *A. proteus*.
2. No "spireme" (Liesche) stage was observed in *A. carolinensis*.
3. No granules (Chalkley and Daniel) were visible at the distal extremities of the spindle fibers at metaphase.
4. During metaphase and anaphase grouping of the distal ends of the polar spindle fibers is not so pronounced in *A. carolinensis*, and during anaphase the chromosome plates are only slightly arched.

SUMMARY

1. It is concluded that the giant amoeba described by Wilson (1900) is not a *Pelomyxa* but belongs to the genus *Amoeba* and should be designated *A. carolinensis*. *Chaos chaos* is considered to be invalid as a name, owing to the fact that it is based on Roesel's inadequate description.

2. Nutritional studies indicate that *A. carolinensis* has food requirements similar to those of *A. proteus* and that these two species react in a similar manner to the same type of food.

3. Nuclear division is described for *A. carolinensis*. The stages are similar to those described for *A. proteus*, and except for smaller size, correlated with a smaller number of chromosomes, the differences are insignificant.

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THE ELIMINATION OF CHROMOSOMES IN THE MEIOTIC DIVISIONS OF BRACHYSTETHUS RUBROMACULATUS DALLAS

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The occurrence of meiotic abnormalities is anything but rare. However, in almost every instance it is the result of mechanical or physiological accidents and therefore sporadic and irregular. Hence, if in a certain species a departure from the normal process always takes a very particular form and is restricted to a certain region of the gonad, it is likely that it is not entirely accidental. If, furthermore, every male or female in the species is affected, it is safe to assume that we are dealing with a basic and well regulated condition, unorthodox though it may be.

The case in question is that of *Brachystethus rubromaculatus*, one of the pentatomid Hemiptera. It holds for all the males of the species. The testis here is composed of four lobes and in the fourth of these (counting from the side where the sperm duct makes its exit) the meiosis follows an abnormal but very definite course. In a way, this is analogous to the case of Loxa (Schrader, 1945a and b), but that is only in the sense that in both the exceptional development takes place in a certain lobe of every testis. The nature of the abnormality is quite different in the two species, as will appear in the account below. My chief interest lies in the possibility that such irregularities may be of use in the analysis of the ordinary mechanism of mitosis and in this as well as several succeeding studies I hope to show that they may throw some light on certain puzzling aspects of the division cycle.

Material and Methods

The gonads of four males and one female were used in the investigation. The insects were collected near Turrialba, Costa Rica, during April and May, 1944. The material was fixed in Sanfelice and sectioned at 5 to 10 μ. Gentian violet, haematoxylin and the Feulgen reaction were used in staining, the haematoxylin being especially useful in the analysis of spindle conditions whereas the Feulgen reaction is indispensable in following the maneuvers of the chromosomes. The main manifestations of the mitosis are so clear that they are well shown by pen and ink drawings—a method which in most other cases does not give a just presentation of spindle conditions.

I take pleasure in expressing my gratitude to Dr. E. N. Bressman and Mr. R. A. Nichols of the Inter-American Institute of Agricultural Sciences for facilitating the work. My special thanks are due to Dr. T. J. Grant of the U. S. Department of Agriculture, whose ready helpfulness did so much to further my researches in Costa Rica.

Normal Spermatogenesis

The testis has only four lobes of which the first three show an orthodox spermatogenesis differing in no essential from that described in so many other pentatomids.

The fourth or "harlequin" lobe has about the same proportions as the rest and does not differ in any discernible way in its general organization.

The size differences among the chromosomes as seen in the spermatogonia are not very great. But it is possible to recognize one large, four medium and one small pair of autosomes. The X is a trifle larger than one of the small autosomes while the Y is the smallest chromosome of the complement (Fig. 1). Identification of the sex chromosomes was checked by examination of oogonial metaphases in the female.

The normal meiosis shows the usual prophase conditions, with the leptotene, synaptotene, pachytene, and diplotene stages followed by the confused period in which chromosomal behavior is difficult to analyze. This is succeeded by a diakinesis marked by the appearance of beautiful tetrads which, as they condense, are distributed around the nuclear periphery. Just before the breakdown of the nuclear membrane this rather even distribution begins to disappear and the chromosomes may even come in contact with each other in small groups of two or three each (Fig. 2). This collocation reaches its height when the membrane disintegrates. All of the chromosomes then huddle together in the middle of the nuclear space only to separate again almost at once to form the equatorial plate. In the latter they follow the characteristic pentatomid arrangement of the first metaphase, with one or both sex chromosomes taking a central position within a ring of autosomal tetrads (Fig. 3).

The sex chromosomes are marked by their heteropycnosis from the leptotene period on. Until the synaptic period they are well separated from each other, but then tend to come together. During the confused period they form a single, rounded chromatin nucleolus and it is not until after the early diakinesis that they once more become independent of each other. They divide equationally in the first, and segregate in the second division, so that the spermatids carry 6 + X or 6 + Y chromosomes (Figs. 3–5).

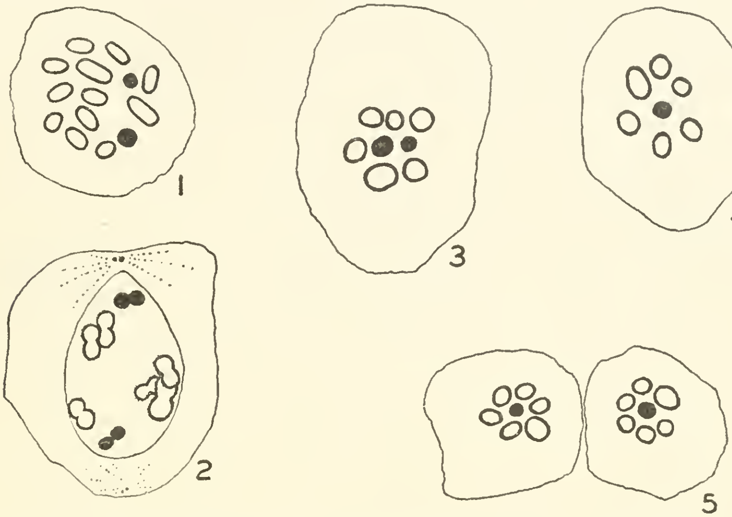
There is no interkinesis and the two centrioles at each pole have already separated to assume their position for the second division before the chromosomes have completed the anaphase movement of the first. This precocious behavior of the centrioles is however also encountered in several other pentatomids and does not interfere with the normal meiotic distribution of the chromosomes except in such extreme cases as Peromatus (Schrader, 1941b).

Abnormal Spermatogenesis

First division

Neither in the spermatogonial nor the meiotic behavior up to the late diakinesis does the harlequin lobe differ from the other three. Six autosomal tetrads are formed which resemble in every way those seen in the normal lobes (Fig. 6). Again, as these tetrads condense into the characteristic dumbbell shaped bodies, they and the sex chromosomes are well distributed around the nuclear periphery.

But then arises a difference in behavior from the normal. Here, too, the last phase prior to membrane disintegration witnesses a coming together of the chromosomes. In this fourth lobe, however, the collocation is both more regular and more pronounced. In nearly all cells the six tetrads form a single chain whose constituents maintain contact with the nuclear periphery. This chain is always more or less equatorially placed (Fig. 7 and 8). The configuration is evidently the resultant of several different forces. Just as in normal cells, the tendency to collocate begins to manifest itself in late diakinesis. But in this fourth lobe the autosomal tetrads are also repelled by the two opposite poles at this early stage. Since the chromosomes are still confined within the nuclear membrane they consequently move into the middle region. The combination of polar repulsion, the tendency to collocate, and adhesion to the still intact nuclear membrane must perforce result in the formation of an equatorial chain.



Drawings were made from haematoxylin preparations, except for Figures 12 and 24. All figures magnified approximately 1400 ×. Autosomes drawn in outline, and sex chromosomes in solid black throughout.

FIGURE A. Normal spermatogenesis. 1. Spermatogonial metaphase; 12 autosomes and X (large) and Y. 2. Late diakinesis; beginning of autosomal clumping. 3. Metaphase of Division I; X and Y in middle. 4. Metaphase of Division II; X superimposed on Y. 5. Telophases of Division II showing two types of spermatids: 6 autosomes + Y, 6 autosomes + X.

As one might expect, there is no constant seriation in such a chain. When the nuclear membrane disintegrates, the chain is converted into an irregular clump just like the clump formed by the tetrads of normal cells that have undergone no such maneuvers (Fig. 9 and 10). Much less frequently is there a formation of two smaller aggregates instead of the single large one. The two sex chromosomes are included in such groupings only by accident and sooner or later they always separate from the autosomes and assume an independent position. This is not necessarily an equatorial one at first (Fig. 8–10).

It is, however, in the establishment of the first metaphase that the most striking departures from an orthodox behavior are evinced. As already stated, the breakdown of the nuclear membrane is followed immediately by the clumping of the autosomal tetrads in the midregion. In many cells this may, however, be halted temporarily if a central core of spindle fibers is formed quickly between the poles. The clumping autosomes may then be applied to these central fibers in a half moon configuration for a moment (Fig. 11). But in any case, before the metaphase spindle has assumed final shape, the autosomal aggregate is shifted out of this general middle region. Almost always this movement seems to be a sudden one and frequently the aggregate comes to rest rather close to the lateral wall of the cell, the direction of the shift being toward the side and never toward the poles. The cell frequently bulges out on the side on which the aggregate is located (Fig. 14). If instead of one aggregate, two smaller ones have been formed, they undergo similar reactions and often become displaced toward opposite sides of the cell (Fig. 13).

The beginning of this autosomal shift sees the X and Y in no definite position although usually in the vicinity of the polar axis. Frequently they are situated at some distance from the equatorial plane and bear neither a constant positional relation to each other nor a mitotic orientation toward the centrioles (Fig. 9 and 10).

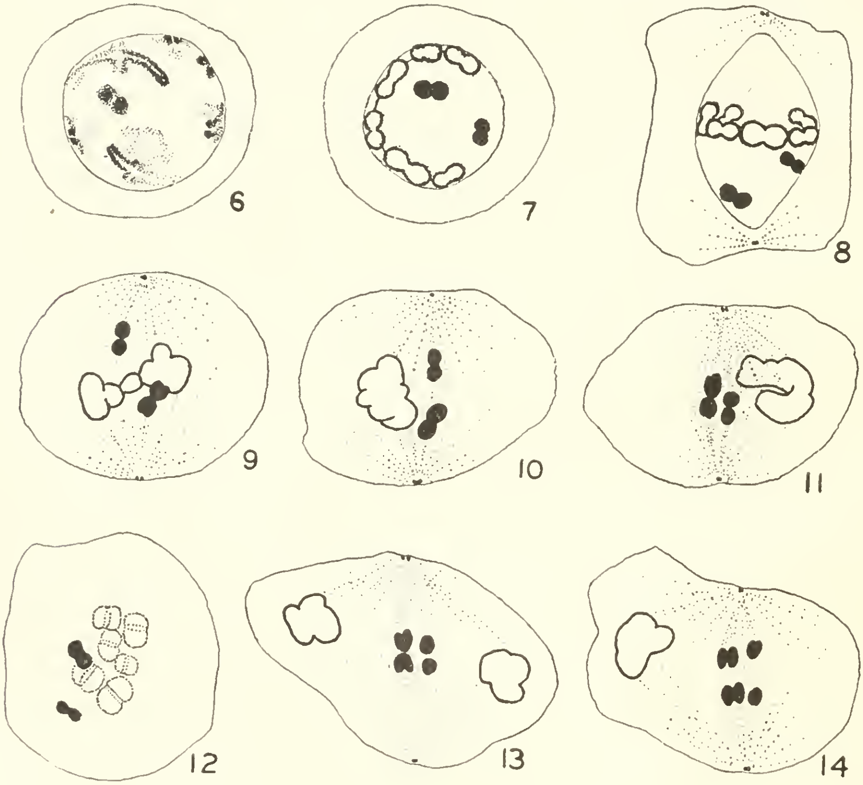


Figure B. Abnormal spermatogenesis. Division I from diakinesis to early anaphase. 6. Mid diakinesis showing tetrads of normal appearance. 7. Late diakinesis in polar view; autosomal tetrads forming equatorial chain. 8. Late diakinesis in side view. 9. Early stage in clumping of autosomal tetrads. 10. Beginning of movement of autosomal aggregate away from polar axis. 11. Displaced autosomal aggregate applied to central portion of spindle in half moon form; X and Y assuming equatorial position. 12. Feulgen preparation corresponding to Figures 9 or 10 showing that tetrads retain their individuality in the aggregate. 13. Autosomes in two aggregates, both shunted out of polar axis. 14. Early anaphase, with X clearly showing "tertiary" split; characteristic displacement of autosomal aggregate.

However this situation is quickly altered and as the autosomal aggregate moves toward the side of the cell, the X and Y approach very close to the polar axis and assume a position in the equator with a definite orientation toward the poles (Fig. 11 and 13).

In all of the hundred or more cells observed at this stage, such a configuration of autosomes and sex chromosomes is always maintained. Since in the prometaphase the two types of chromosomes form one general group albeit not always in contact with each other, it is clear that the later separation is not an accidental one. The unusual shift of the autosomes is equivalent to an actual extrusion from the mid-region of the cell.

Despite their distance from the polar axis, the autosomes continue to be connected with both poles by chromosomal spindle fibers. This is at first glance rather surprising since with ordinary stains like gentian violet and haematoxylin the autosomal aggregate appears as a solid, structureless mass which plainly suggests degeneration. In good Feulgen preparations, however, it becomes clear that the autosomal tetrads have by no means lost their individuality. All six of them can be easily distinguished, lying in a substance which does not stain with Feulgen and thus reveals the individual chromosomes (Fig. 12). This substance, which with other stains becomes just as dark as the chromosomes themselves, resembles the material surrounding the sex chromosomes of certain reduviid Hemiptera (Troedsson, 1944) and may also be akin to the "flocculent, whey-like coagulum" which envelops some regions of the chromosomes in Olfersia (Cooper, 1944). In Brachystethus it is evidently formed when the chromosomes clump at prometaphase and it persists through both meiotic divisions.

In the middle of the cell, between the two poles, there is a well-formed spindle of normal length, which however is smaller in diameter than the spindle of normal cells. This accommodates the two sex chromosomes, which are located in the middle of the spindle substance just as they would be if the autosomes were free to form a ring around them.

Despite the presence of the chromosomal fibers which connect the autosomes with the poles, the autosomal aggregate behaves more or less like an inert mass in the mitosis that follows. While the sex chromosomes undergo an equational division and approach the poles, the autosomes near the periphery of the cell undergo no movement (Fig. 14–17). It is only when the dividing cell elongates and becomes narrow that the autosomal clump is moved toward the midline (Fig. 18). If it there comes in contact with the expanding interzonal region or "Stemmkörper" it may be swept along into one of the two daughter cells, but very often it is not until the cleavage constriction is being completed that the aggregate is definitely included in either of the resulting cells (Fig. 19 and 20). Apparently the chromosomal fibers exert little or no traction at this time. So far as one can tell, the inclusion of the autosomes in either daughter cell occurs entirely at random.

It is of interest to note that although in its essentials the equational division of the sex chromosomes is always accomplished successfully, there are certain features that distinguish it from the corresponding process in normal cells. In the first place, the sex chromosomes and especially the X clearly show a tertiary split already at this first metaphase—a split which is not utilized until the first somatic division in the egg (Fig. 13–15). This is sometimes indicated in normal cells also, but never so strikingly as here where it appears after all three of the stains used. The second feature lies in the anaphase behavior of the sex chromosomes. Although both start out from an equatorial position, the early anaphase often shows the chromatids of the Y much closer to the poles than those of the X (Fig. 15). In other words, the Y precedes the X in the poleward movement. When there is such a disparity of movement, there is frequently a shift during mid anaphase through which these four chromatids are all brought into the exact polar axis in a single line. Since as just stated, the chromatids of the Y move more quickly, they constitute the extremities in this tandem arrangement, whereas the two X chromatids are in between (Fig. 16). Such a seriation strongly resembles that which characterizes the second division of the coccid Protortonia (Schrader, 1931), and there is little doubt that similar forces are involved. This anaphasic shift does not affect the result of the division; one X and one Y chromatid go into each of the resulting second spermatocytes.

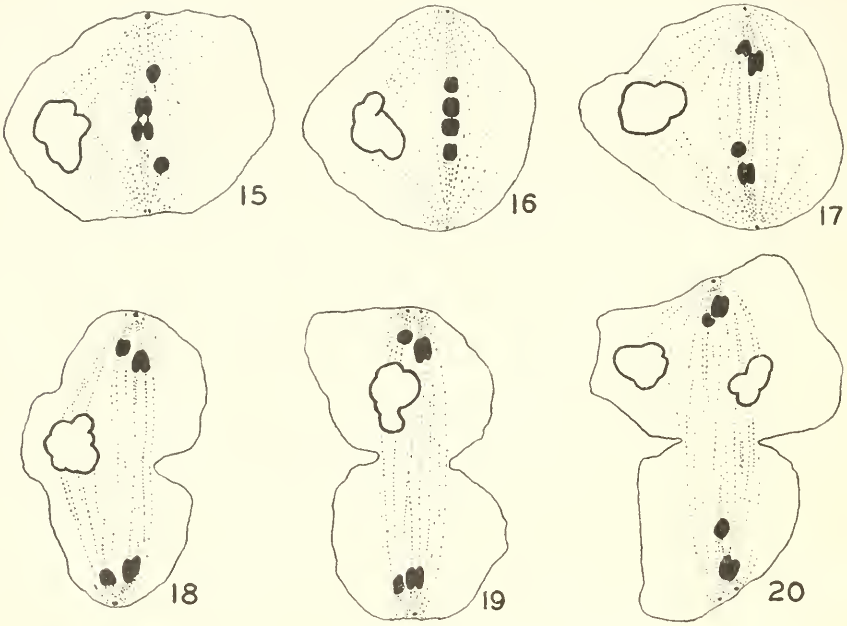


Figure C. Abnormal spermatogenesis. Division I, anaphase and telophase. 15. Early anaphase with Y preceding to the poles. 16. Early anaphase; X chromatids have moved into polar axis between separating Y chromatids. 17. Mid anaphase. 18. Late anaphase; autosomal aggregate returning to axial region. 19. Early telophase; an X and a Y at each pole with the autosomal aggregate included in upper cell. 20. Late telophase with two autosomal aggregates both included in upper cell.

Thus in the majority of cases, two types of second spermatocytes are produced: one carries an X and a Y, as well as the clumped autosomes; the other contains only the two sex chromosomes (Fig. 19). If the autosomes have been aggregated in two masses before metaphase, these may both go to the same pole or to opposite poles, apparently at random (Fig. 20). In no case is there a division of the individual tetrads.

Second division

As in normal cells, the centriole at each pole of the first spindle is divided and the two daughter centrioles separate some time before the division has been finished (Fig. 21). Each moves through an arc of 90° and the new polar axis for the second division is therefore at right angles to the first. Even while still in telophase, the two sex chromosomes often respond to the new poles and move toward them (Fig. 21). However, this precocious movement is soon reversed and the X and Y then come together in the middle of the new spindle in their orthodox "touch and go" pairing (Fig. 22 and 28) and it is only following this that they separate in the regular segregation toward opposite poles. There is no indication of any interkinesis.

The autosomal aggregate may at first remain in the general region where it has entered the cell upon completion of the first division, but in other cases it approaches closely to the two sex chromosomes that are near the new polar axis (Fig. 21). Such an approach is only temporary, however, and before the second division is initiated the aggregate is always extruded from this middle region just as it was in the first division (Fig. 23). The configuration of the second division thus almost duplicates that of the first.

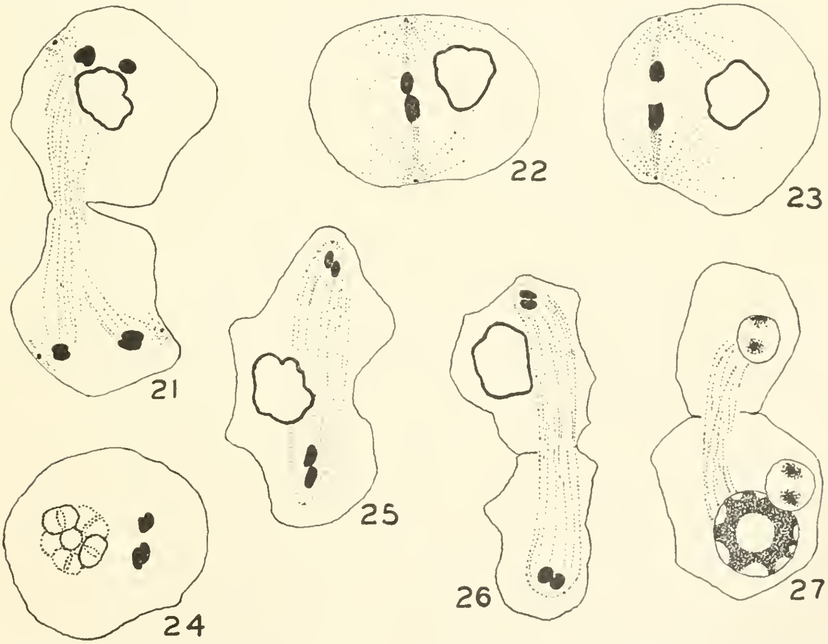


FIGURE D. Abnormal spermatogenesis. Division II of cell containing autosomal aggregate (all drawings except Fig. 21 show X below Y). 21. Beginning of Division II; X and Y reacting to new position of poles. 22. Touch and go pairing of X and Y; autosomal aggregate shunted away from polar axis. 23. Early anaphase; X and Y going to opposite poles; autosomal aggregate in characteristic displacement. 24. Feulgen preparation showing that tetrads still appear unaltered in early phase of Division II. 25. Anaphase; autosomal aggregate returning toward polar axis. 26. Telophase; autosomal aggregate included in cell with Y. 27. Spermatids; upper cell carries only a micronucleus with the Y half chromatids; lower cell with micronucleus containing X half chromatids and autosomal nucleus.

The autosomal tetrads are still intact and are still imbedded in the substance that stains intensely with gentian violet and haematoxylin. However, the aggregate is usually smaller than it was just prior to the first division, a fact that results from a greater crowding of the tetrads, as can be seen in Feulgen preparations (compare Fig. 24 with Fig. 12). Again, chromosomal fibers are formed (Fig. 22), although the aggregate seems to be moved about as a more or less passive body (Fig. 25 and 26). In short, just as in the first division it is only the sex chromosomes that are involved in the regular mitotic mechanism. But now, as in normal second divisions, the process is reductional and the resulting cells receive only an X or a Y (Fig. 26 and 30). The autosomes if present are once more included in either cell as a group, and so far as one can tell, on the basis of chance (Fig. 23–26).

The spermatids that result from these two peculiar divisions thus either carry only one sex chromosome (the X or the Y) or else they contain the autosomal aggregate in addition. In other words, there are four main types of spermatids: X; Y; X + autosomes; Y + autosomes (Fig. 26 and 30).

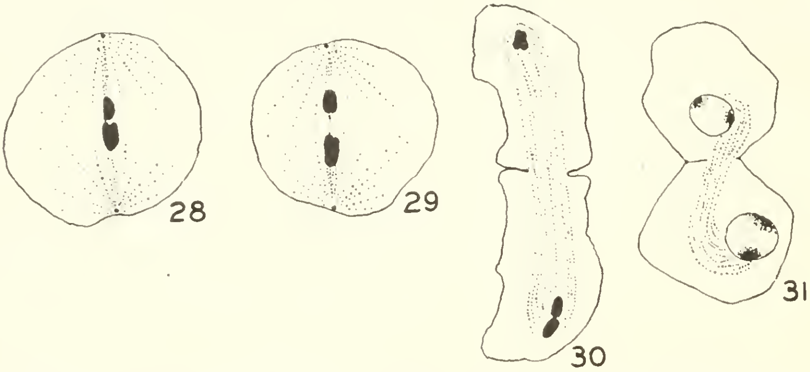


FIGURE E. Abnormal spermatogenesis. Division II of cell lacking autosomes (all drawings show X below Y). 28. Touch and go pairing of X and Y. 29. Early anaphase. 30. Late telophase. 31. Spermatids; upper cell with micronucleus containing the two Y half chromatids, lower with two X half chromatids.

If the autosomes are gathered in two instead of the usual single group prior to the first division, the resulting spermatids may of course carry intermediate numbers of chromosomes. But for the great majority of cells they are transmitted in a single aggregate. Throughout there is no attempt at a division of the individual autosomal tetrad, and it is as an aggregate of tetrads that the autosomes enter the spermatid. Obviously there is some factor which interferes with their mitotic mechanism—a factor which interferes in no way with that of the sex chromosomes. The latter behave just as they do in the normal cells of neighboring lobes of the testis.

Spermateleosis

The first steps after the telophase of the second division parallel the normal course of events. The transformation of the autosomal mass into the spherical nuclear structure shown in Figure 27 is peculiar, though in later stages it approaches closely the condition seen in the normal spermatid. In these abnormal cells however, the sex chromosome at first forms a micronucleus which is distinct from the larger autosomal nucleus (Fig. 27). But sooner or later, the micronucleus becomes applied to the large nucleus and gradually merges with it.

When, as in most cases, a single sex chromosome constitutes the only chromosomal constituent of the spermatid, its behavior differs in no way from that in cells containing the autosomes as well. In either case a tiny nucleus is formed by each sex chromosome and in this the constituent half chromatids separate to form two distinct chromosomal bodies. (Fig. 27 and 31). This precocious separation is not surprising when it is remembered that already in the first metaphase these half chromatids can be clearly identified as such.

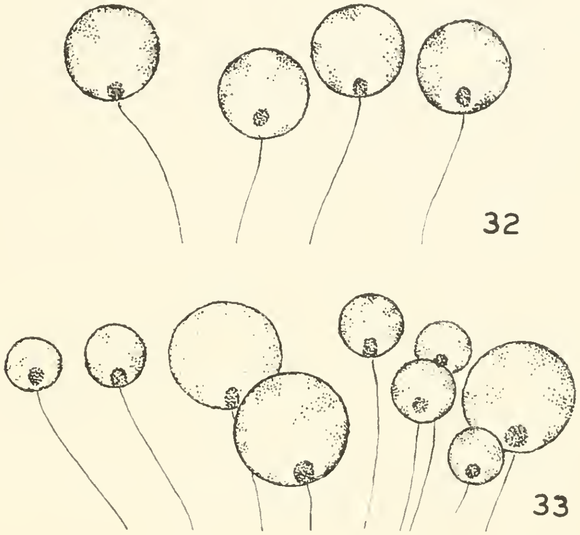


FIGURE F. Spermatids before elongation. 32. Normal spermatids shown to indicate size. 33. Abnormal spermatids at stage corresponding to Figure 32. Largest spermatids contain all autosomes as well as either X or Y. Smaller spermatids carry either X or Y; latter is the smallest.

The process of transformation into the sperm seems at first alike in all spermatids and homologous to that observed in the normal lobes. This is true whether they carry only a single chromosome or the autosomes as well. As might be expected, the former are distinctly smaller than the corresponding normal cells and nuclei (Fig. 32 and 33); the latter on the other hand are markedly larger. It is also interesting to note that it is often possible to distinguish between the small sperms carrying either an X or a Y. The latter are the smaller of the two (Fig. 33). However, when the rounded sperm heads begin to elongate, those which carry only a sex chromosome begin to stain less intensely than either normal or "autosomal" abnormal sperms, and as the elongation into the typical tenuous sperm head continues it becomes more and more difficult to trace their further fate even in Feulgen preparations. It is likely that the "sex chromosome sperms" never attain the final stages of sperm formation. However, this cannot be made certain without smear preparations for even in very thick sections the long sperm heads are practically always cut and it is impossible to decide whether one is dealing with an abnormally small sperm or only a portion of a larger sperm. The fate of the giant sperms, however, is subject to no such doubt. They continue their development to the fully formed stage, enter the sperm duct, and are there mingled with the normal sperms from the normal lobes.

General Considerations

The cytological features that characterize this abnormal development are thus of the most striking sort. The most obvious one lies in the clumping of the autosomes. A temporary collocation of chromosomes is of course encountered as a normal occurrence in many species, but is there, confined—as it is in the normal lobes of Brachystethus—to a very brief, almost momentary period immediately after the breakdown of the nuclear membrane in prometaphase. In Brachystethus there is some tendency toward a collocation of autosomes even before this event. But in the abnormal lobe, the clump that is finally formed is not resolved again, and this condition is maintained through both meiotic divisions and into sperm formation. Moreover this collocation of chromosomes is so intimate that only a Feulgen preparation reveals that there has not been an actual fusion.

Although the further behavior of this autosomal aggregate suggests strongly that it is shunted about more or less like a passive body during cell division, it nevertheless evinces certain reactions which indicate that it is not completely inert. In diakinesis, these autosomal tetrads duplicate the behavior of normal tetrads in establishing contact with the nuclear periphery; toward the end of the diakinetic period they evince a reaction to the two poles in taking up an equatorial position in contact with the nuclear membrane; after the disappearance of the latter they form chromosomal fibers to the poles; and almost simultaneously they move out of the midregion of the cell with a suddenness that bespeaks a forcible displacement.

It is possible and even likely that this movement toward the side of the cell is due to the same centriolar repulsion which forces the chromosomes into the equatorial plane while they are still held within the confines of the nuclear membrane. An influence of centrioles on the chromosomes prior to the dissolution of the nuclear membrane is observed in certain other forms also (for instance in Anisolabis, Schrader, 1941a). But not often is it exerted so as to bring about an equatorial placement at so early a stage, even though a role in the later final formation of the equatorial plate is frequently assigned to it.

It will be realized, however, that even in normal cases, additional factors must function to restrict the chromosomes to the middle of the equatorial plane. Repulsion from the two poles alone cannot do that. It is possible that the chromosomes are thus confined to the midregion simply because of surface tension conditions that prevail in the spindle body at the time of metaphase. The escape of the autosomes in the present case might then be attributed to abnormalities in the spindle, say to untoward alterations in the timing of the normal viscosity changes. But that is clearly not the entire explanation since the autosomes are not accompanied in their displacement by the two sex chromosomes. The latter remain in the spindle in a quite orthodox position. Since the cytoplasm, the centriolar forces, and the general spindle conditions are identical for both autosomes and sex chromosomes, it is therefore in the chromosomes themselves that the explanation must be sought. More specifically, the question to be solved is why the autosomes do not respond to the forces which at metaphase counteract the influence of the centers and confine the chromosomes to the middle of the equatorial plane.

The abnormal condition of the autosomes is not indicated by any striking behavior during the prophase. There is only a more pronounced tendency to assume an equatorial position at prometaphase and the formation of chains that are absent in normal cells. Also, after the aggregate has been formed, Feulgen preparations show the component tetrads to be somewhat swollen and less intensely stained than are the prometaphase tetrads. This probably indicates the first step in a return to a diffuse state. Such a regressive condition may in some way be responsible for the special maneuvers of the autosomes, for the X and Y chromosomes which during this time remain fully condensed behave just like the sex chromosomes of normal cells. But it is not certain that this would suffice as an explanation since in addition to the clumping of the autosomes one must also account for their elimination from the midregion and their failure to take the first steps in division. For it must be remembered that the initial separation of daughter chromosomes is an autonomous process which is independent of spindle action. Nevertheless, although the Brachystethus tetrads appear ready for such a step and the line of demarcation between the paired chromosomes is clearly marked and complete, no separation ever occurs. Either there is no mutual repulsion or else the pellicle and the achromatic constituents of the tetrads prevent the normal division. In any case it is clear that some of the basic reactions of the chromosomes have been altered.

It is natural to seek a parallel in other cases already on record. The investigation of certain echinoderm hybrids by Baltzer (1910) is especially pertinent. Baltzer found that in certain of these crosses, the paternal chromosomes are eliminated during cleavage. There, too, is a tendency for such chromosomes to clump, and there also a formation of chromosomal spindle fibers occurs nevertheless. Likewise there is sometimes a lateral elimination of these chromosomes although they usually simply lag in the middle of the spindle. However, all this does not occur until the anaphase is well advanced and the various configurations are by no means as constant as they are in Brachystethus. After a careful analysis, Baltzer concluded that it is the chromosomes themselves rather than the plasma that is responsible for the elimination. That such a conclusion is warranted for the case of Brachystethus, as well, has already been pointed out. The difference in behavior between the autosomes and the sex chromosomes in the same plasma makes it unavoidable.

The elimination of chromosomes consequent on a loss of their kinetochores is observed regularly in certain molluscs (Pollister, and Pollister, 1943). Such an explanation is worthy of serious consideration in the case of Brachystethus. However, there can be only a partial loss of the kinetochore activity because some chromosomal fibers are evidently still being formed. Since we are dealing here with a diffuse kinetochore, such a partial loss is easily conceivable, but it must be confessed that the akinetic chromosomes of molluscs present very different elimination pictures than those seen in Brachystethus.

The conditions in such coccids as Phenacoccus (Hughes-Schrader, 1935) may also be germane to the present case. There, too, certain chromosomes betray a tendency to clump, and it is these chromosomes that become inert and degenerate within one or two succeeding divisions. Nevertheless, these chromosomes form normal chromosomal fibers.

But all these other instances are themselves in need of further explanation. The only conclusion that protrudes itself in such an analysis is that the abnormality is to be sought in the basic organization of these forms and is not a superficial and accidental one. The alterations that are involved primarily affect the chromosomes and influence their reactions to each other and to the mitotic mechanism.

Evolutionary Aspects

It is very questionable whether spermatids carrying only an X or a Y chromosome ever develop into mature sperms. That, however, is not true of the giant sperms which in addition to a sex chromosome carry the full set of autosomes unaffected by any meiotic mitosis (i.e., four times the number of autosomes contained in a normal sperm). But though these large sperms enter the sperm duct and mingle with the normal sperms, it is doubtful whether they ever become functional in the hereditary sense. Of the seven specimens of Brachystethus that I have examined, none was marked by unusual morphological features such as confidently might be expected if a sperm nucleus with a quadruple set of autosomes joins the haploid nucleus of a normal egg.

But if these numerous giant sperms play no direct role in the hereditary mechanism of the species, it becomes a matter of wonder that such an extensive development of abnormal gametes could have withstood the effects of natural selection. For it must be remembered that the testis of Brachystethus has only four lobes and if the sperms of one of these do not function in the genetic determination of the embryo, we are confronted with a prodigious waste which is added to that which occurs normally in the reproduction of most male animals.

Such a waste is paralleled in several species of Loxa (Schrader, 1945a and b) where there is likewise an abnormal lobe in every testis. Although the nature of the aberrancy is different in the two cases, they are similar in that both encompass the production of a huge number of sperms which carry many times the normal number of chromosomes. It seems strange that two processes so diverse in their abnormalities should culminate in gametes whose general characters are so similar.

The explanation may lie in the fact that though these large sperms take no part in the direct control of the heredity of the species, they may nevertheless be important in its welfare. Since they are normal in every respect except size, it is more than likely that they enter the egg like the normal sperms. It also must be recalled that polyspermy is almost universal in the fertilization of insect eggs and that in many species the number of supernumerary sperms is very high. The entrance of genetically inert sperms would probably offer no difficulties in the regular fertilization process and a union between two normal pronuclei would proceed as usual.

It is clear from evidence in marine eggs that the breakdown of gamete nuclei releases substances which have a far reaching influence on the reactions in the egg. It is likely that such substances are present in abnormally large quantities in the giant sperms and that therefore their possibly beneficial effects are increased. Again, if the developing embryo utilizes the nucleo-proteins that are brought in by supernumerary sperms, an advantage might well accrue to a species which has such substances available in the unusually large amounts that are represented in the giant sperms. In short, the latter may play a not inconsiderable role in the embryology.

If that is the case, the harlequin lobe would in no sense be a useless organ. The development of such a lobe in the testes of two genera that are taxonomically as diverse as Brachystethus and Loxa may well have been due to a nongenetic but metabolic advantage that is thereby conferred on the developing embryos.

SUMMARY

1. The fourth lobe in all testes of the pentatomid *Brachystethus rubromaculatus* shows an aberrant meiosis of a very definite character.

2. The first indication of this aberrancy is found in a chain formation and clumping of the autosomal tetrads just prior to metaphase. The two sex chromosomes are not included in this aggregation of autosomes.

3. At metaphase the X and the Y take a normal position in the spindle but the aggregate of autosomes is shunted laterally out of the middle region of the cell, away from the polar axis.

4. The two sex chromosomes divide equationally in the first division while the autosomal aggregate passes unaltered to either pole.

5. In the second division the X and Y again behave as in a normal meiosis and separate to opposite poles. The autosomal aggregate, if present, passes to either pole apparently at random.

6. Since the clumped autosomal tetrads pass undivided through both divisions while the sex chromosomes behave normally, the following four main types of spermatids are produced: X; Y; X + autosomes; Y + autosomes.

7. The mechanics and evolution of so constant an aberrancy are discussed.

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AN INVESTIGATION OF CROSS STRIATIONS AND MYOSIN FILAMENTS IN MUSCLE*

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Introduction

Present information on muscle structure is of two rather distinct types, that derived from histological studies through direct observation with the light microscope, and that deduced from indirect methods such as involve polarized light microscopy, streaming double refraction of myosin solutions or x-ray diffraction. Histological studies have always been limited by the inadequate resolving power of light microscopes; the indirect methods have been limited by the fact that they can supply information only in proportion to the correctness of the necessary assumptions. Although valuable knowledge has resulted from both types of approach, there is a lack of definite information on structures below the limit of light microscope resolution. The electron microscope provides the possibility of extending direct observation into a range of dimensions reached hitherto only by indirect methods and conjecture. Since success in biological electron microscopy depends to a large extent on the development of techniques suitable for the material, these initial observations may be expanded as new techniques are developed.

It is impossible in the present space to summarize the previous research on muscle or the ideas put forth concerning its structure; reference to the literature will be limited to those papers which provide a basis for the present study. A general review has been presented by Fenn (1945) while the histological aspects have been reviewed by Jordan (1933). Polarized light investigations have been reviewed by Schmidt (1937) and by Weber (1934). Results of investigations of streaming double refraction in myosin solutions may be found in the papers of Edsall (1930, 1942), von Muralt and Edsall (1930), Mehl (1938) and Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham and Shen (1944). Astbury and Dickinson (1940) and Astbury (1942) described wide-angle x-ray diagrams of muscle and myosin, and Bear (1944, 1945) described small-angle x-ray patterns of various muscles. A discussion of muscle physics has been presented in a review by Ramsey (1944).

In the past, the electron microscope has been used very little in the study of muscle structure. Richards, Anderson, and Hance (1942) have shown electron micrographs of a wedge-cut section of cockroach striated muscle in which each darker band can be seen to consist of three components. Sections of muscle tissue cut by another method were examined by Sjöstrand (1943) but the electron micrographs bear no obvious relation to known structures in muscle. In both these studies the object was primarily to investigate sectioning techniques for electron microscopy rather than to study muscle itself.

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* The substance of this paper was presented at the meetings of the Electron Microscope Society of America on December 1, 1945.

Ardenne and Weber (1941) published electron micrographs of filamentous myosin particles, which are undoubtedly to be identified with the asymmetrical particles responsible for the streaming birefringence phenomena in myosin sols. Hall, Jakus, and Schmitt (1945) have studied another fibrous protein from molluscan muscles and correlated the structure with the x-ray diffraction results described by Bear (1944).

ELECTRON MICROSCOPE OBSERVATIONS OF MYOFIBRILS

Preparation of specimens

A primary difficulty in the study of striated muscle with the electron microscope is the preparation of the tissue thin enough to be partially transparent to the electron beam. In view of the difficulties connected with present microtome techniques, a preparative method was sought in another direction. One procedure which has been used previously depends on the tendency of many materials to fragment along natural cleavage boundaries. If such tendency to natural cleavage exists, it may be assisted by chemical or physical means or both. Thus collagenous tissue may be separated into fine fibrils by mechanical "teasing" and the separation is facilitated by weak acid (Schmitt, Hall, and Jakus, 1942). Such methods of fragmentation are applicable to many materials and are readily adapted to striated muscle.

Fresh muscle is fixed in 10 per cent formalin, cut into small pieces and subjected to mechanical agitation in a Waring Blendor. When the resulting suspension is lightly centrifuged to throw down the larger fragments, the supernatant exhibits the characteristic sheen associated with fibrous suspensions. It consists mostly of thin fibrils which may be washed and applied directly to a conventional electron microscope specimen grid with collodion membrane. These fibrils are to be identified with the myofibrils, or sarcostyles, of the muscle fiber.

Most of the observations to be described were made on fibrils from the leg muscles of frog and rabbit. The muscles of lobster and scallop (striated portion) were found to contain relatively large fibrils which were usually quite opaque in the electron microscope. It was necessary to apply fixatives to all these muscles in order to obtain intact fibrils. Wing muscles of the fly, on the other hand, could be teased apart in weak saline solution without previous fixation.

Most myofibrils from frog muscle have widths between 0.5 and 1.0 μ, although a few may be found as wide as 3.0 μ or as narrow as 0.2 μ. They appear to be ribbon-shaped on the electron microscope specimen holder but it is not possible to say whether this shape results from forces produced during drying or whether it represents the form of the myofibril in the intact tissue.

Although most of the structural features of the myofibril can be observed without the use of stain, contrast in the image may be increased by the application of phosphotungstic acid to the specimen (0.1 per cent solution at pH 3–5 for about one minute). The staining procedure is found to be particularly useful in increasing the contrast of the myosin filaments.

An RCA electron microscope Type B with accelerating voltage raised to 65 KV was used throughout the study. A higher voltage would probably be advantageous for this type of material but is not readily obtainable from the standard power supply.

Unstained fibrils

Unstained myofibrils from striated muscle are characterized by a succession of transverse bands of varying density, the main features of which can readily be identified with the bands previously described in histological studies (Jordan, 1933). An electron micrograph of a typical fibril from a slightly stretched frog sartorius is shown in Figure 1, together with the histological designations. The repeating unit, or sarcomere, is bounded terminally by a narrow dense band usually referred to as the *Z* membrane or telophragma. This band is in the center of a light region, the *I* (or *J*) band, so called because it is relatively isotropic. Contiguous with *I* is the sharply-defined *A* (or *Q*) band which is optically anisotropic and has a higher scattering power for the electron beam than does *I*. In some myofibrils the *H* disc (or median disc of Hensen) appears as a lighter region in the middle of the *A* band (Fig. 1). Bisecting the *A*, or the *H* disc if it is present, is the narrow dark *M* band (or mesophragma). There may also appear, in either half of the *I* band, a relatively dark band designated as *N* in Figure 2. These bands are not always present and have been considered by some cytologists to be artifacts resulting from the lining up of granules. In the present study they have been noted in rabbit muscles but are either faint or absent in frog muscles. The clarity of their appearance in rabbit fibrils leaves no doubt as to their existence in this formalin-fixed material.

Myofibrils from rabbit muscle usually show two to four fine cross bands symmetrically disposed with respect to the *M* band and near it. They are beyond light microscope resolution, being about 0.1 μ from center to center, and represent a periodic variation in the dense material of the *A* band.

In favorable cases fibrils can be seen to consist of longitudinal myosin filaments which are difficult to resolve in unstained fibrils except where they fray out at the edges. They are visible in various parts of Figures 1 and 2.

Stained fibrils

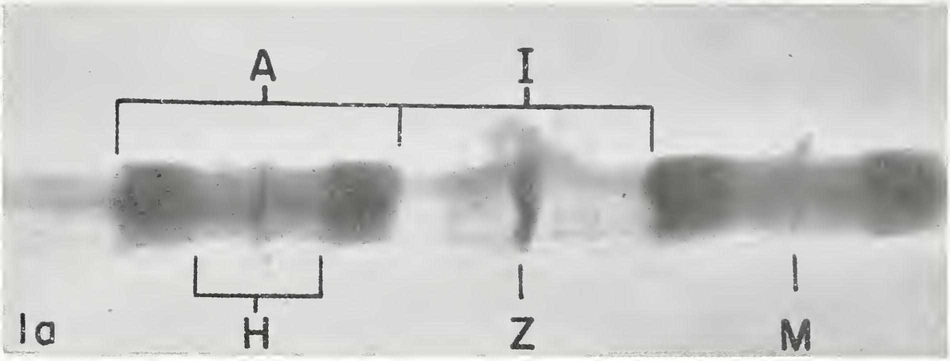
Phosphotungstic acid combines with the cross bands roughly in proportion to their intrinsic density and serves to increase the contrast of the structures described above. Segments of stretched myofibrils stained with phosphotungstic acid are shown in Figure 3. The *A* band is stained more heavily than is the *I* band, and the *M* and *Z* bands appear quite opaque. When the *H* disc is present, it absorbs less stain than does the remainder of *A*.

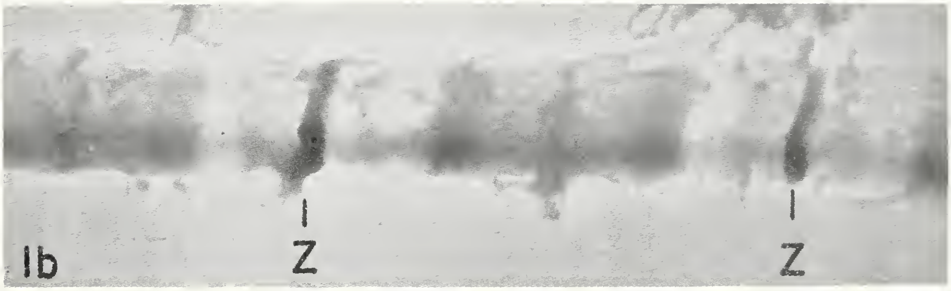
Besides accentuating the transverse bands, phosphotungstic acid enhances the contrast of the longitudinal myosin filaments. These range in width from 50 to 250 Å and extend continuously, and in relatively straight lines, through both *A* and *I* bands. Although the filaments are usually indistinguishable within the dense *Z* bands, they can be traced through several successive sarcomeres when the *Z* bands are partially disintegrated. In the *A* band the filaments are relatively dense, sharply defined, and almost parallel in orientation. In the *I* band they are less perfectly aligned and of lower density. The higher density of the *A* band appears to result from the higher density of the component filaments.

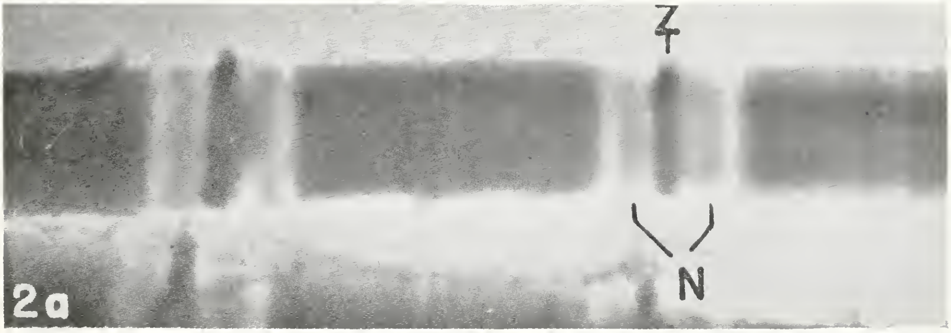
FIGURE 1. Myofibrils from frog sartorius, stretched about 30 per cent, unstained. × 25,000. All myofibrils shown in this and subsequent figures were fixed in 10 per cent formalin.

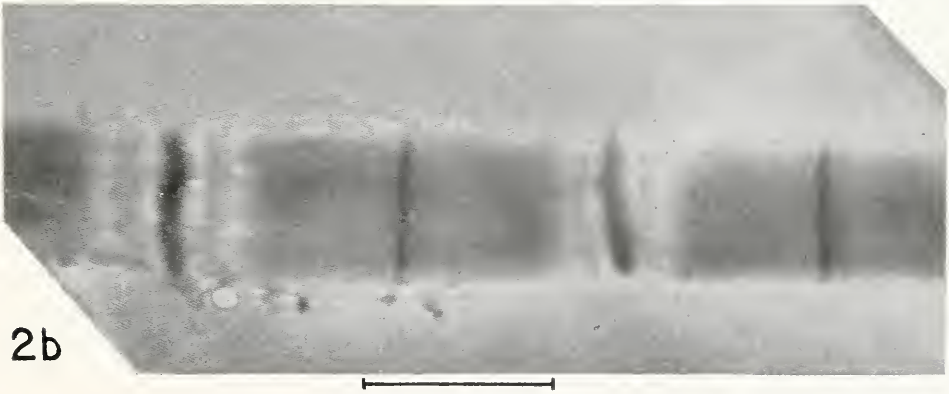
FIGURE 2. Myofibrils from rabbit leg muscle, unstained. × 25,000.

PLATE I









In both bands, the myosin filaments present a knotted or beaded appearance with frequent constrictions and variations in density. The nodes are often almost equidistant along the filament and, in some regions of the fibril, they may be aligned laterally to produce a fine cross striation with a period of about 400 Å in the direction of the fibril axis. However, this feature has not been found sufficiently reproducible or regular to be designated as a periodic spacing in the nature of those found in collagen and in clam muscle fibrils.

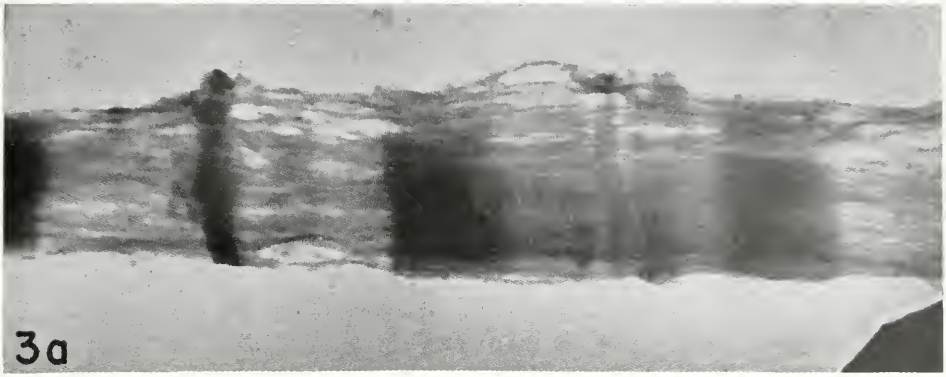
The higher density of the *A* band in electron micrographs of unstained fibrils is in accord with the fact that the refractive index of *A* is higher than that of *I*. Since the myosin filaments pass continuously through both bands, it may be concluded that some other substances occur in much higher concentration in *A* than in *I*. This conclusion is in agreement with the observations of Scott (1932), who found a higher concentration of salts in the *A* band, and of Macallum (1905), who demonstrated a distribution of potassium within the *A* band which is remarkably like the distribution of unstained density as seen in Figure 1. Thus it seems quite evident that the *A* band contains, besides myosin filaments, some substance characterized by a relatively high concentration of salts. This material will be designated as the "*A* substance". Phosphotungstic acid is apparently absorbed in proportion to the density of the *A* substance. It should be noted that phosphotungstic acid forms an insoluble complex with potassium and has been used in the quantitative determination of potassium by Rieben and Van Slyke (1944). This does not mean that the phosphotungstic acid locates potassium specifically for it may react with numerous substances; nevertheless, the observed absorption of this stain in the *A* band is consistent with the conclusion that the *A* substance does contain a relatively high concentration of potassium. That the *A* substance is closely associated with the myosin filaments is indicated by the lack of any observable quantity of interfilamentary material in frayed *A* bands.

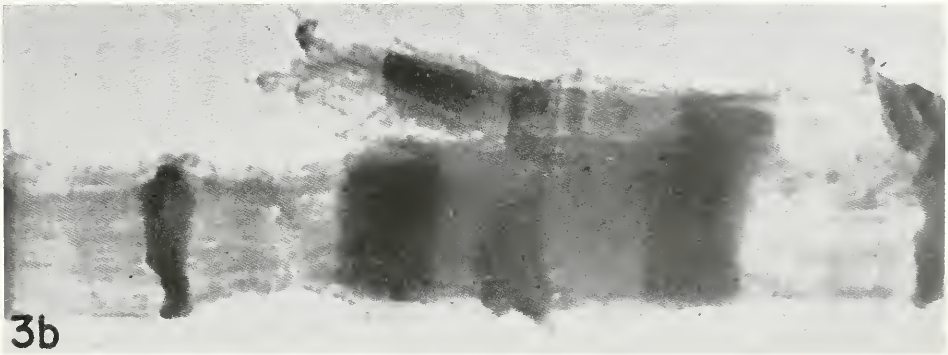
The sharp boundary between the *A* and *I* bands is noteworthy since there is no apparent membrane or other structure to confine the *A* substance. Owing to the limited resolution of the light microscope it had been concluded (Schmidt, 1937) that the transition between the *A* and *I* bands is not abrupt but gradual. Likewise there is no evidence in electron micrographs for any envelope or limiting membrane around the myofibril.

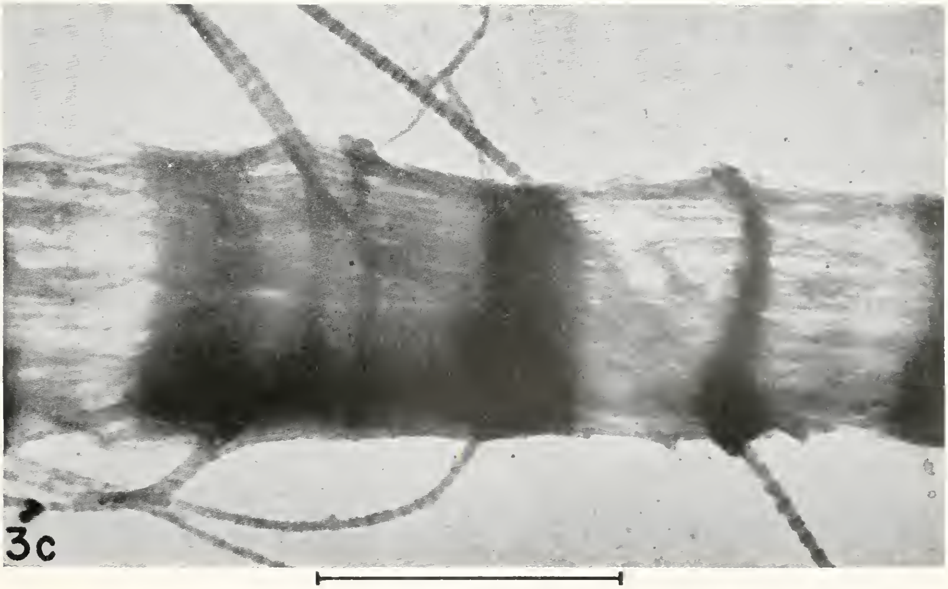
One of the most prominent histological features of striated muscle is the *Z* membrane, the nature of which has been the subject of much discussion in the past. In electron micrographs the *Z* membrane appears to be amorphous material of high staining affinity which cements the myosin filaments together in this region. Frequently in frayed myofibrils the filaments separate laterally but adhere to one another in the region of *Z* and also at *M*. Furthermore, there is a tendency for the filaments to break at *Z* and sometimes at *M*. It has been postulated that the *Z* membrane takes the form of an annular ring about the fibril (see Liang, 1936). Although this could be true, there is no unequivocal electron microscope evidence for such a conclusion. The *Z* membrane appears to consist of interfilamentary material present throughout the fibril and not limited to the periphery. It is definitely not collagenous as was suggested by Häggqvist (1931).

FIGURE 3. Myofibrils from frog sartorius, stretched about 30 per cent, stained with phosphotungstic acid; (*c*) shows collagen fibrils. × 40,000.

Plate II


3a


3b


3c

Collagen in muscle

In the preparation of specimens no effort was made to separate the collagen, which occurs in skeletal muscle in appreciable quantity. However, there is no difficulty in identifying collagen since it has a regular spacing of about 640 Å and displays a characteristic fine structure after staining with phosphotungstic acid (Schmitt, Hall, and Jakus, 1945). Several stained collagen fibrils are visible in Figure 3c. It has been observed that collagen fibrils from rabbit and frog muscle are quite uniform in width (about 500 Å), but the relation of this protein to the muscle structure is not evident in present electron micrographs because of the extensive fragmentation of the muscle.

Structural alterations in extension and contraction

Although evidence is not yet sufficient to permit a complete description of the structural alterations associated with extension and contraction, some observations have been made and may be described. Extended sarcomeres were obtained by stretching an excised frog sartorious and fixing it at the stretched length. In some instances the muscle was stimulated electrically during fixation to produce isometric contraction. In the absence of the electrical stimulus, the fixative itself provided a weakly stimulating effect. In other experiments, excised muscles were placed in formalin without restraint, thus producing a state of weak isotonic contraction. Strong isotonic contraction was produced by electrical stimulation of excised muscles before and during fixation. Myofibrils from these muscles were prepared for electron microscope observation in the manner described.

The high concentration of *A* substance toward the ends of the *A* band, resulting in the appearance of the *H* disc, occurs consistently in myofibrils from muscles which have been stimulated (electrically or by the fixative) while held in a state of extension (Figs. 1 and 3). Muscles which are free from tension and are stimulated to contract during fixation do not in general show a division of the *A* band. Myofibrils from such muscles are shown in Figure 2.

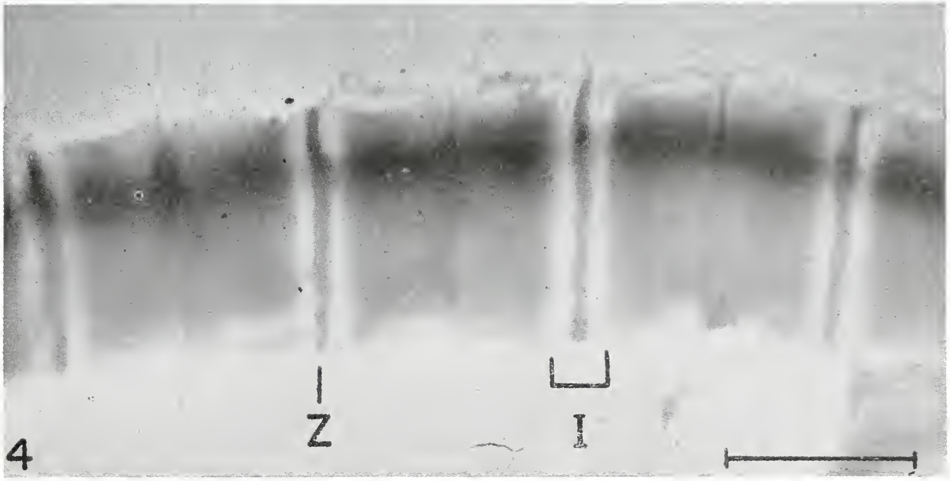
In the myofibrils of muscles which were stretched up to 130 per cent of rest length and fixed at this length, the *A* bands are about the same length as those in muscles which had contracted slightly as a result of fixation. Thus the increase in sarcomere length in stretched and fixed muscles is due mainly to an increase in the length of the *I* band. This observed relative constancy in the length of the *A* band during extension is in agreement with the findings of Buchtal, Knappeis, and Lindhard (1936) who concluded that the *I* band has a relatively low modulus of elasticity.

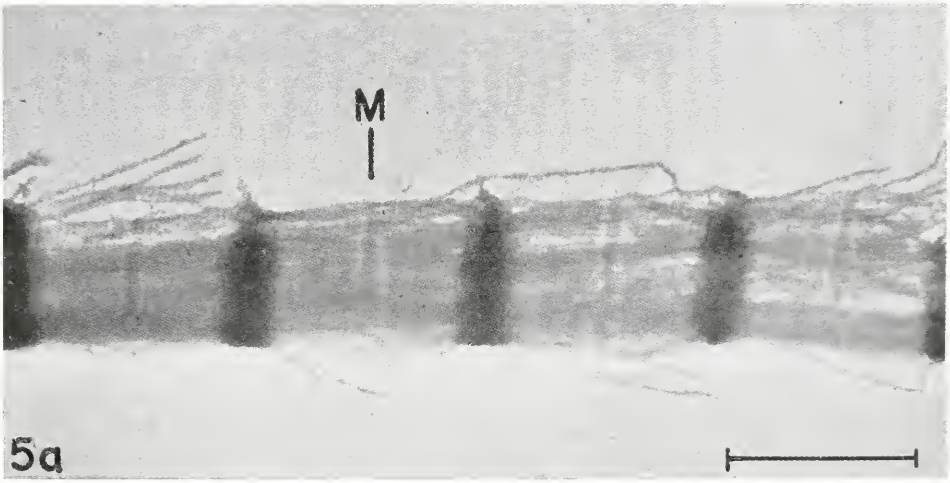
Electron micrographs of myofibrils from muscles fixed in a state of strong contraction show sarcomere lengths down to about 1 μ as compared with about 2 μ in relaxed or weakly contracted muscles. In general the observed sarcomere structure is of two types. The first type, shown in Figure 4, consists of a very narrow *I* band and a quite solid *A* band in which the median *H* disc is absent or very faint. The sarcomere length in Figure 4 is about 1.5 μ, which is about 25 per cent less than the average length in relaxed muscles. The second type of sarcomere structure

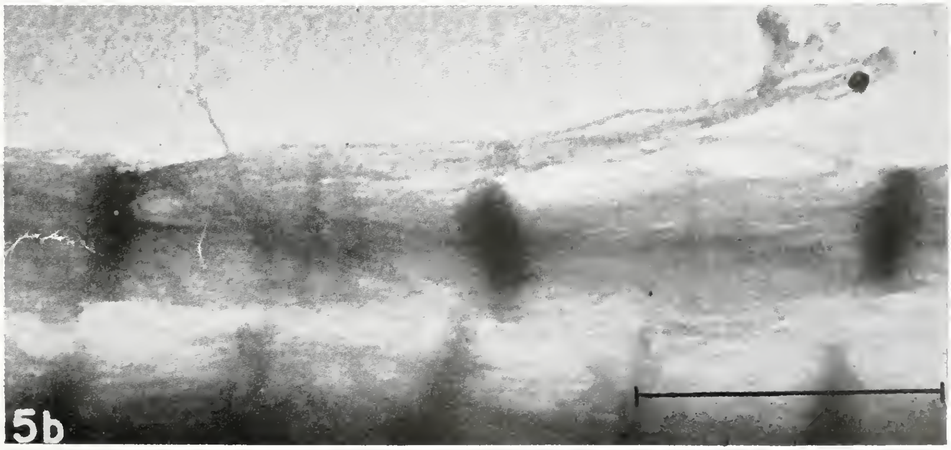
FIGURE 4. Myofibril from frog sartorius contracted by electrical stimulation, stained with phosphotungstic acid. × 25,000.

FIGURE 5. Myofibrils from frog sartorius, strongly contracted by electrical stimulation. (*a*) × 25,000 (*b*) × 40,000.

Plate III







found in strongly contracted muscles is shown in Figure 5. Sarcomere lengths in this case are about 1.2 μ, which is about 60 per cent of the average relaxed length. The *I* band is no longer visible and *Z* appears somewhat wider and more poorly defined than in less strongly contracted fibrils. The region between *M* and *Z* is of uniform density and presumably contains a uniform concentration of *A* substance. In occasional fibrils the *Z* and *M* are distinguishable only with difficulty. Such fibrils may represent a transition stage between the two types of fibrils described. If this is the case, the second type of fibril might result from the accumulation, around the *Z*, of *A* substance which had migrated away from *M*. This stage apparently corresponds to the striation reversal described by Jordan (1933) and earlier by Rollett (1891), who refers to this densely staining *Z* as the *C*, or contraction band.

One aspect of particular significance in strongly contracted sarcomeres is the relative straightness of the myosin filaments, as seen in Figure 5. Since the contracted sarcomeres are as little as 50 per cent of the relaxed length, this can only mean that the filaments themselves shorten in contraction. Similarly, in extension the filaments must individually lengthen. Changes in sarcomere length are not to be associated with any gross spiralling or folding of filaments in the order of dimensions visible in the electron microscope. The myosin filaments are the smallest visible contractile units.

Isolated Myosin Filaments

Myosin may be extracted from muscle in weakly alkaline salt solutions and these extracts have been shown to contain filaments visible in the electron microscope (Ardenne and Weber, 1941). In order to determine whether visible differences exist between different myosins, a quantitative study of filaments from various muscles was undertaken.

Extracts of myosin were prepared by a method essentially similar to that described by Greenstein and Edsall (1940). Muscles were removed from the animal immediately after death, trimmed, cut into small pieces and blended in a Waring Blendor with about ten parts of a cold solution of KCl (0.5 M) and $NaHCO_3$ (0.03 M). The suspension was stirred mechanically, at about 4° C., for periods of time varying from seven to twenty-four hours and strained through several layers of closely-woven cheese-cloth. The filtrate, showing strong double refraction of flow, was poured into 8 to 10 volumes of cold distilled water, with constant stirring, and the precipitate which formed was allowed to settle overnight in the cold. Further concentration was accomplished by centrifugation, after removal of the supernatant. The precipitated myosin was washed with cold distilled water, and redissolved by adding powdered KCl crystals to a concentration of about 0.45 M. To further purify the myosin the precipitation and solution was repeated once or twice.

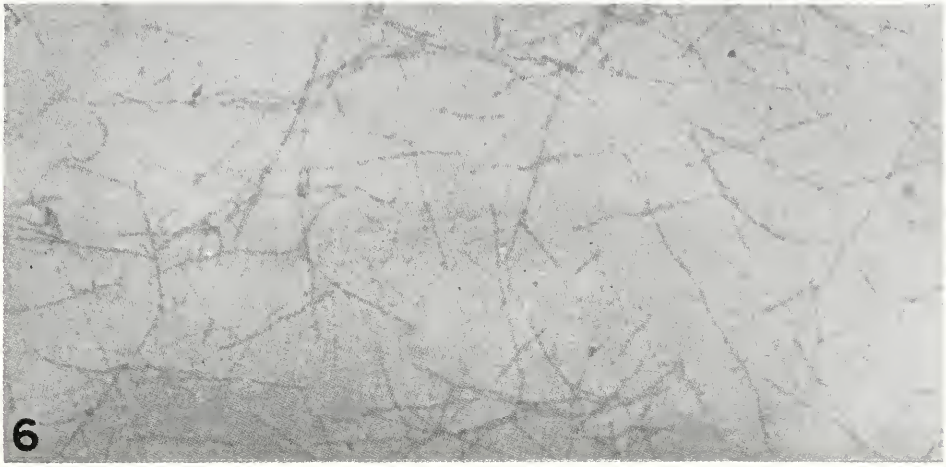
For electron microscope examination, the myosin preparation was diluted to the desired concentration with the KCl–$NaHCO_3$ solution used in the extraction and centrifuged to remove any undissolved protein. A droplet of the solution was

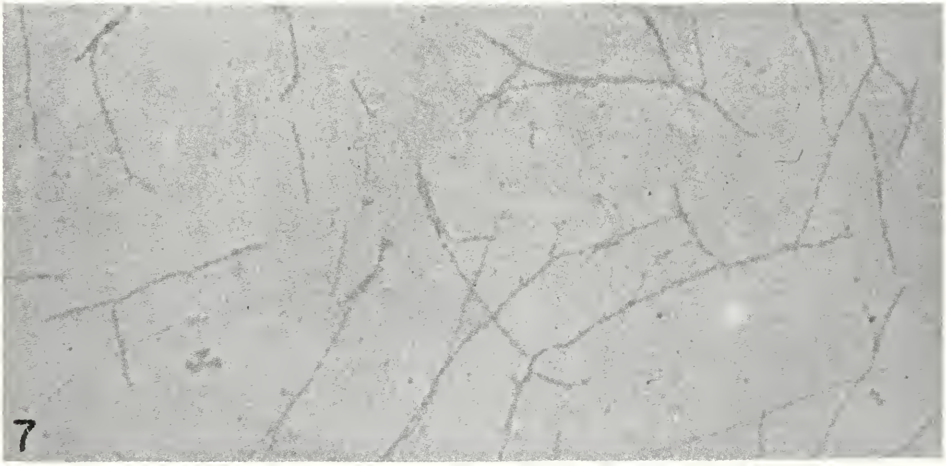
FIGURE 6. Myosin filaments from rabbit leg muscle. × 30,000.

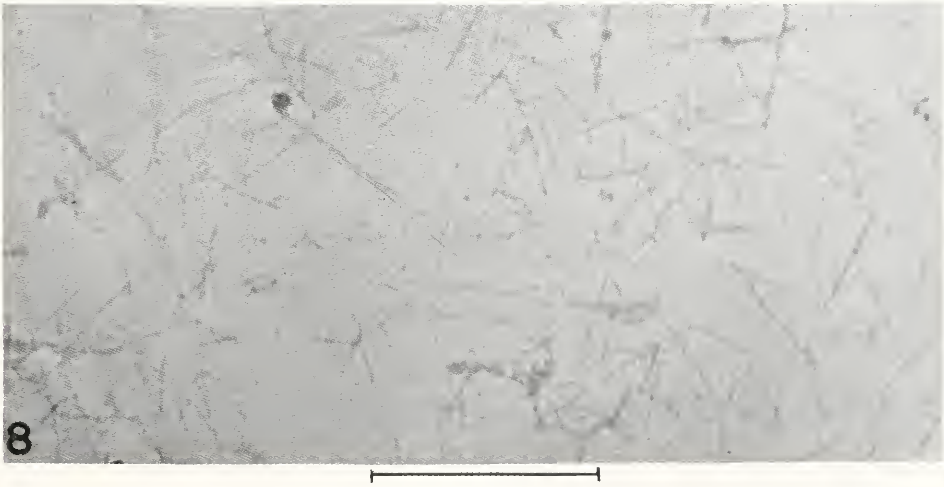
FIGURE 7. Myosin filaments from lobster abdominal muscle. × 30,000.

FIGURE 8. Myosin filaments from clam (Mya arenaria) adductor muscle. × 30,000.

Plate IV







placed on the supporting film of the specimen grid, the excess removed by blotting, and the film washed with Edsall's solution. The adhering filaments were then fixed and stained, for about one minute, with 0.1 per cent phospho-12-tungstic acid (pH 3 to 5) and washed briefly with water to remove the uncombined phosphotungstic acid. Unstained filaments are poorly defined because of low contrast and, in the absence of the stabilizing effect of the heavy metal ion, appear to be adversely affected by the drying process.

Myosin extracts were made from the muscles of rabbit leg, frog leg, lobster abdomen, scallop adductor (striated part) and clam (Mya) adductor (classified as smooth muscle). Electron micrographs of such myosin preparations show slender filaments similar to those observed in intact myofibrils and resembling those shown by Ardenne and Weber (1941). The filaments have varying lengths and widths and display no tendency to branch or split longitudinally. They are rough in appearance and show fluctuations in density along their length. However, there is no evidence of any regular structural variation which could be interpreted as a significant periodicity. There are no observable changes in density which can be correlated with the *A* and *I* bands, which indicates that the *A* substance has probably been washed out during extraction. This general appearance is very much the same for myosin filaments from the five different animal forms. Typical electron micrographs of myosin filaments from rabbit, lobster, and clam are shown in Figures 6, 7, and 8 respectively.

A statistical study of lengths and widths of myosin filaments from the five selected forms was made. Measurements were taken directly from enlarged prints and in any given print all filaments were measured which could be discerned as complete and individual. Widths can be measured only approximately because of the smallness of this dimension and also because of the roughness of contour. The uncertainty of measurement, however, is significantly smaller than the spread in widths. Results are shown in Figure 9. Each interval in the plot of widths contains the same number of measurable increments and each set of measurements represents about 300 filaments.

TABLE I

Dimensions of myosin filaments

Muscle source	Average width	Average length
Rabbit leg	120 Å	4100 Å
Frog leg	140 Å	4100 Å
Lobster abdomen	140 Å	6800 Å
Scallop adductor, striated	130 Å	5000 Å
Clam (Mya) adductor, smooth	150 Å	3100 Å

Nearly all measured widths fall into a narrow range between 50 and 300 Å. There may be some filaments having widths below 50 Å which were not observed because of the resolution and contrast limitations of the electron microscope. However, the quality of the background in the electron micrographs and the fact that all distributions fall off toward small widths indicate that no great quantity of the myosin occurs in this range in electron microscope specimens as prepared.

Between one animal form and another there is very little variation in magnitudes or distribution of widths. There is a slight difference in the averages as shown in Table I, but it is so small as to be scarcely significant.

Filament widths were also measured from electron micrographs of intact myofibrils from frog muscles. The distribution plot resembles very closely the corre-

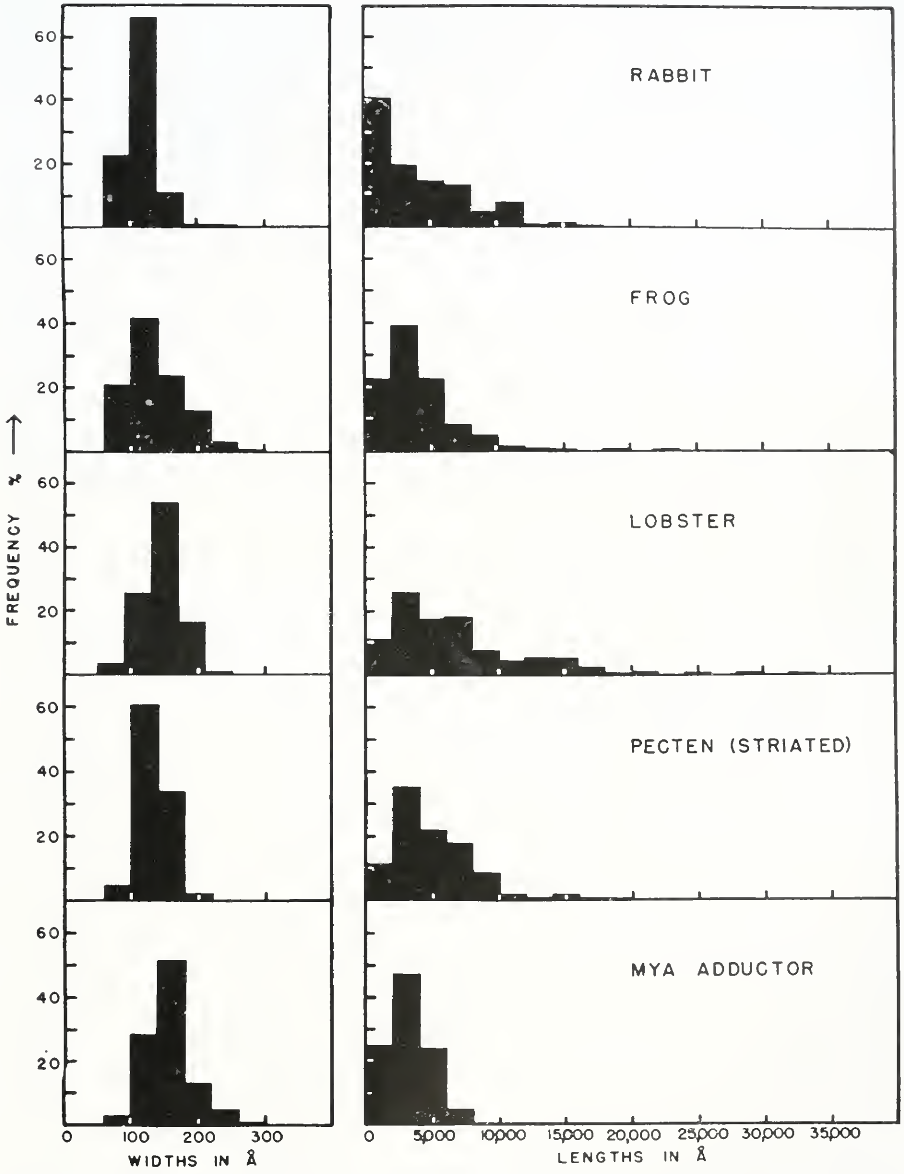


FIGURE 9. Distribution of lengths and widths of myosin filaments from muscles of various animal forms.

sponding plot for frog shown in Figure 9, thus indicating that there is no great difference in the width of filaments in electron micrographs of intact fibrils and myosin extracts.

In comparison to the fair constancy of widths there is a wide distribution of lengths. Most of the measured lengths are below 15,000 Å. Filaments from lobster muscle are in general significantly longer than the others, while filaments from Mya aductor, which is the only smooth muscle studied, are significantly shorter. As a result of the wide range of lengths there is only rough significance in the number-averages given in Table I. The distribution plots are reasonably reproducible under similar conditions.

It is evident from the statistical results that myosin extracts of this type do not constitute a monodisperse system and there is no justification for referring to the filaments as molecules. Widths of filaments apparently do not alter appreciably during extraction. The lengths, however, bear no relation to any observable dimensions in the intact myofibrils. Since most of the lengths represented in the plots of Figure 9 are considerably shorter than are the corresponding sarcomeres, it is apparent that the myosin filaments have been broken at random into shorter segments during the extraction procedure.

Since the myosin filaments seen with the electron microscope are to be identified with the asymmetrical particles responsible for the streaming double refraction of myosin solutions, it is pertinent to compare the results of the methods where possible. Mehl (1938) reported the length of rabbit myosin as 8,500 Å while Edsall (1942) gives a figure of 12,600Å. The second figure is near the upper limit of the distribution plot for rabbit in Figure 9 while the first falls within the range of lengths representing the bulk of the protein. Although no figures have been reported on the other muscles used here, Edsall and Mehl (1940) have described lobster myosin as being more viscous than rabbit myosin at equal concentration and strongly birefringent, which is in qualitative agreement with the electron microscope observation that lobster preparations contain significantly longer fibrils than do those from rabbit.

Ardenne and Weber (1941) do not give the source of the myosin extract from which they made electron micrographs. They state that the filament widths are from 50 to 100 Å although no measurements are tabulated and the micrographs used as illustration contain filaments at least 200 Å in width. The myosin filaments within the limited field of the micrographs which these authors have shown are apparently similar to those described here.

Paramyosin

In a recent publication Hall, Jakus, and Schmitt (1945) described the structure of a fibrous muscle globulin which is present in appreciable amount in molluscan smooth muscles, and correlated the observed structure with the x-ray patterns obtained by Bear (1944) from the same material. Since this protein can be identified by electron microscope observation and x-ray diffraction it merits a distinguishing name and is therefore designated as *paramyosin*. Although paramyosin

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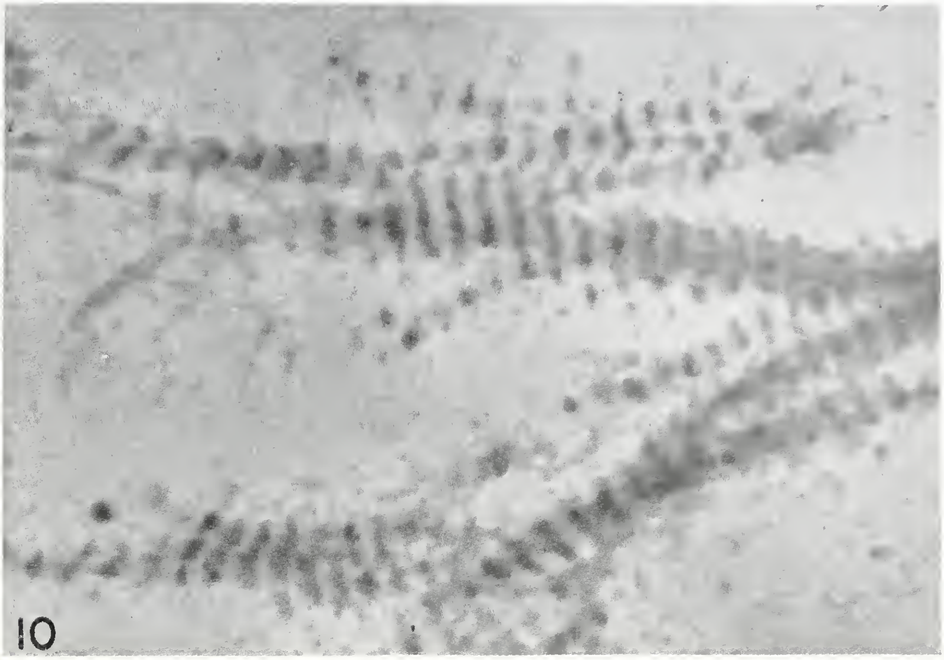
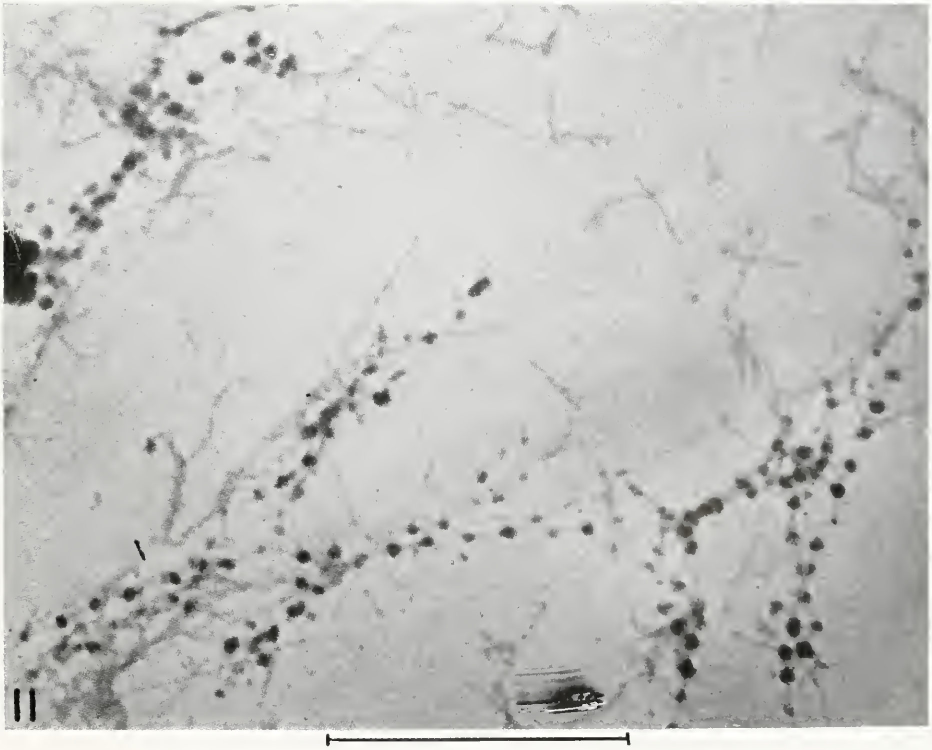
FIGURE 10. Regular structures from clam adductor muscle after dispersion in Edsall's solution. × 40,000.

FIGURE 11. Fragments from dispersed clam muscle. Darkly staining particles are adhering to fine filaments, presumably myosin. × 40,000.

PLATE V





resembles myosin in some respects, there are definite differences between the two proteins. Paramyosin contains a characteristic axis spacing of about 145 Å. Paramyosin has not been detected in striated muscle while myosin is apparently common to all muscles. Furthermore, the typical needle-shaped fibrils of paramyosin disintegrate in Edsall's solution (0.5 M KCl, 0.03 M $NaHCO_3$) while the myosin filaments remain essentially the same as in fixed intact muscle except as they may be broken into shorter lengths during extraction.

Clam adductor muscles

The adductor muscle of the clam differs from the other muscles investigated in that it is classified histologically as "smooth" muscle and contains a large quantity of the fibrous protein, paramyosin. Electron microscope observation has failed to reveal any large periodicity resembling the characteristically banded sarcomere of striated muscle. However, if clam muscle is dispersed in Edsall's solution and centrifuged, there are thrown down fibrous aggregates to which large particles adhere. A typical micrograph of this type of material is shown in Figure 10. The structure consists essentially of bundles of fine filaments (presumably myosin) and darkly staining nodules producing a cross striation with a period of about 1,100 Å. In Figure 11 the bundles have been dispersed, revealing the individual dark nodules which adhere to the fine filaments at more or less regular intervals. The attachment of the dense component at regular intervals along myosin bundles (Fig. 10) is suggestive of a rudimentary structure analogous to the *Z* membrane and sarcomere of striated muscle. As yet, the significance of this structure is not known.

Discussion

With respect to the existence and disposition of the cross striations, the electron microscope observations on fixed muscle, stained and unstained, are in close agreement with the results obtained with the light microscope (see Jordan, 1933). Confirmatory evidence is presented concerning the *N* bands, *H* discs and the nature of "striation reversal" in strong contraction. Additional information has been added by virtue of the high resolution of the electron microscope. Thus it is possible to say definitely that the *Z* band is not collagenous as suggested by Häggqvist (1931) and to observe directly the myosin filaments and their relation to previously determined structures.

Of fundamental importance is the observation that the myosin filaments extend continuously through the fibril in relatively parallel straight lines. There is no marked disorientation of the filaments in either *A* or *I* bands of fibrils in any state of contraction. Although the optical anisotropy of the *A* band led to the postulate that it contains asymmetric myosin particles, there has always been some doubt as to whether the relatively isotropic *I* band consists of myosin in unoriented state or of some other protein (Weber, 1934). It is now clear that the *I* band consists mainly of well oriented myosin filaments and it is not possible to account for the low birefringence on the basis of gross disorientation. Possibly the difference in anisotropy between the *A* and *I* bands is to be attributed to differences in orientation within the filaments. The only obvious alternative explanation is that the low birefringence in the *I* band is due to partial compensation of the birefringence of the myosin filaments by other components.

No evidence has been found in this investigation for the existence, either in myofibrils or in extracted myosin, of "rodlets" of the specific dimensions postulated by Weber (1934) from polarized light and diffusion experiments. Theoretical difficulties underlying Weber's calculations have been pointed out by Frey-Wyssling (1940) and Schmitt (1944).

Electron micrographs of isolated myosin filaments show that in width and general appearance the filaments are similar to those seen in fixed intact myofibrils. Although myosin filaments from whatever source are essentially the same, the statistical study indicates that they occur in various lengths and are not to be designated as discrete "myosin molecules". However, the length of rabbit myosin reported by Mehl (1938) from streaming double refraction studies is reasonably close to the weight-average length calculated from the distribution curve in Figure 9. No reliable measurement of width is available for comparison with the electron microscope results.

Since myosin filaments in intact fibrils are continuous, the wide range of lengths found in myosin suspensions is noteworthy. It appears that the filaments, while little changed in width, are broken more or less at random during the extraction procedure. The longest filaments approach the sarcomere length and there may be significance in the fact that lobster muscle yields longer filaments than does frog muscle and also has the longer sarcomere. The absence of filaments longer than their corresponding sarcomere is consistent with the observed tendency of the filaments to break at *Z*.

Ziff and Moore (1944), following an extraction procedure similar to that used here, state that their myosin solutions contain a homogeneous substance which forms sharp boundaries in electrophoresis and sedimentation. If homogeneity is meant to denote constancy in particle length, this conclusion is in disagreement with the electron microscope results. In considering the apparent inconsistency it should be noted that, in the electrophoresis and sedimentation of rod-shaped particles of nearly constant diameter, a sharp boundary does not necessarily indicate a constant length. Schramm and Weber (1942) reported that a small fraction of the myosin in extracts has a much higher sedimentation constant than that of the predominant component. No evidence for such a distinct heavy component has been found in the present study unless it represents aggregates of myosin filaments.

The "molecular weight" of myosin has been estimated by Weber and Stöver (1933) to be $0.6 - 1.2 \times 10^6$ and by Ziff and Moore (1944) to be 3.9×10^6. If a particle weight is calculated from the average dimensions of the filaments in rabbit myosin from Table I, using a density of 1.3 and assuming a circular cross section, the result is 36×10^6. The results differ by at least a whole order of magnitude. In view of the fair agreement as to filament lengths from streaming birefringence and electron microscope observations, the discrepancy in particle weight is difficult to understand. It may be that the cross section of filaments dried on the supporting film is not circular as assumed in the electron microscope calculation, but it is doubtful whether this assumption could introduce an error large enough to account for the discrepancy. In any event, the significance of all such calculations is questionable inasmuch as the extracts contain particles of widely differing dimensions, in no sense to be considered as molecular entities.

Bear has reported small-angle x-ray diffractions from various muscles. One set of diffractions (Bear, 1944) has been correlated with the structure of paramyosin fibrils, as determined from electron micrographs (Hall, Jakus, and Schmitt, 1945). This set of diffractions has not been obtained from any striated muscle.

In addition Bear (1945) has reported a second set of small-angle diffractions obtained from a variety of muscles, both smooth and striated. The wide occurrence of this pattern is strongly suggestive that the diffractions originate in the myosin component but it has not been possible to identify in the electron microscope the structure responsible for the diffractions. Furthermore, since the meridional diffractions are orders of 27 Å, it is doubtful whether the structure can be observed directly. Another feature of the diffractions is the occurrence of a periodicity estimated by Bear to be between 350 and 420 Å. Although this dimension is quite large enough for electron microscope resolution, it appears that the pattern is somewhat like the paramyosin pattern in that the large periodicity cannot be discerned unless the axis spacing (27 Å) is well resolved. The fine banded appearance frequently observed in myofibrils is of about the same order of magnitude as the large periodicity, but this may be fortuitous. The x-ray data also indicate a lateral periodicity of about 115 Å which should be large enough for electron miscroscope observation. Although the *average* width of myosin filaments is quite close to this figure, the significance, if any, of this coincidence remains to be determined.

Since the myosin filaments are observed to pursue a straight course through the *A* and *I* bands in fibrils from contracted as well as relaxed muscles, it may be concluded that contractility is a property of the individual filaments in their normal environment in muscle. It seems probable that alterations in length and tension depend on changes within the filaments in response to changes in the chemical environment. In seeking a description of the contractile mechanism, due consideration must be given to the role played by the *A* substance and the adenosine triphosphate. The electron microscope technique provides a promising method for studying these structures and processes. It may be expected that further correlation of the x-ray, electron microscope, polarization optical and chemical evidence will contribute greatly to an understanding of the nature of contractility.

Summary

1. Electron micrographs were made of myofibrils isolated from frog and other skeletal muscles fixed in formalin. The structure with respect to the location and disposition of the principal cross striations is in good agreement with that previously determined from histological studies.

2. The myofibrils are composed of bundles of myosin filaments ranging in width from about 50 to 250 Å and extending continuously and in relatively straight lines through the isotropic and anisotropic bands in both the extended and contracted states. The anisotropic bands also contain material of high electron scattering power and affinity for phosphotungstic acid. The distribution of this "*A* substance" changes with contraction in characteristic fashion. The evidence indicates that the myosin filaments are the contractile units.

3. While the myosin filaments have an indefinite length in the intact fibril, they are fragmented extensively during extraction in weakly alkaline salt solutions (method of Greenstein and Edsall). Filaments from such extracts have fairly uniform widths (50 to 250 Å) but highly variable lengths, in general below 15,000 Å. Filaments from rabbit, frog, lobster, scallop and clam muscles are similar in appear-

ance; widths are fairly uniform but lengths vary significantly from one form to another.

4. The relation of these findings to physical chemical data previously obtained by others on similar myosin extracts is discussed.

5. In the one smooth muscle examined (clam adductor) no striations comparable to those of skeletal muscle fibrils were found. However, a regular structure with a period of about 1,100 Å was observed.

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EFFECT OF WATER CURRENTS UPON THE ATTACHMENT AND GROWTH OF BARNACLES [1]

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Introduction

There exists a considerable body of published work dealing with peculiarities in the distribution of sessile marine invertebrates in general, and, although experimental evidence is incomplete some speculation has been offered as to the manner in which their distribution is limited by various factors of the environment which influence their attachment and growth. The object of the present studies was to investigate the action of the water current factor by experimental methods, with particular reference to the species of barnacles which are most abundant in the Miami area.

Much of the earlier work on sessile marine animals has been reviewed by Mc Dougall (1943), and need not be considered here in detail. A bibliography of investigations dealing with sessile organisms from the point of view of ship fouling is given by Neu (1933), and an excellent general account of marine fouling by Visscher (1928) who does not, however, include more than a brief reference to the effect of water currents.

The effects of environmental factors have been deduced from the results of ecological surveys by a number of investigators. Stephenson and his co-workers, in a series of publications entitled *The South African Zone and Its Relation to Water Currents* (see bibliography), Fischer-Piette (1928, 1928a), Pierron and Huang (1926), and Prennant and Teissier (1924) have described the populations of rocky shores and the distribution of sessile organisms in general, but with scanty reference only to the relation between barnacle attachment and the velocity of water currents. Stephenson and Bright (1938) and Stephenson and du Toit (1937), however, note the absence of certain species of barnacles from surfaces exposed to heavy wave action.

The possibility of water movement influencing barnacle attachment is suggested by Fischer-Piette (1932) in his reports on surveys of the shores of the English Channel. Shelford and Towler (1925), Towler (1930), and Newcombe (1935) have paid particular attention to barnacles in associations of Balanus-Littorina and Balanus-Mytilus and draw attention to the more luxuriant growth of barnacles exposed to strong water currents, particularly in shallow water. The role of water currents is also mentioned briefly in accounts of ecological surveys by Rice (1930), Moore (1935a), and Moore and Kitching (1939), who stress the part played by them in the provision of an adequate food supply, as well as the adverse effects of wave action.

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[1] These experiments were conducted while the author was engaged by the Woods Hole Oceanographic Institution in investigations under contract with the Bureau of Ships, Navy Department, which has given permission to publish the results. The opinions contained herein are those of the author and do not necessarily reflect the official opinion of the Navy Department or of the Naval service at large.

[2] Contribution number 301 from the Woods Hole Oceanographic Institution.

Experimental investigation has yielded more direct information. Moore (1933) studied the effect of currents upon the attachment of cyprid larvae with particular regard to orientation, but gives no estimate of current velocities which limit attachment. The related phenomena of wave action are considered by Moore in a separate paper (1939).

Evidence as to the effect of water currents is also afforded by the study of ships and other objects moving through the water. The economic importance of ship-bottom fouling has stimulated this kind of observation. Heutschel (1934) and Neu (1932) state that vessels collect barnacles most readily when in harbor and when subjected to relatively less water movement. Visscher (1930) further observes that barnacles are usually killed during ocean passages of about 500 miles. Hiraga (1934) showed experimentally that, in the laboratory, 10 to 17 day old barnacles were lost during a five day exposure of test planks to water currents, whereas twenty-four day old barnacles remained alive and intact. In a later paper of Visscher (1938) he concludes that barnacles are not distributed by ships. This is contrary to the views of Fischer-Piette (1929, 1935) who expresses the opinion that *Balanus amphitrite* found by him on the coast of France, was brought from the tropics on the hulls on ships. Later observations by Phelps (1942) on test panels support the conclusion that excessive water currents inhibit the attachment and growth of barnacles.

The extensive observations of McDougall on the pile fauna at Beaufort include the results of exposing various types of experimental collecting apparatus. His experiments on the effect of water currents indicate that fewer barnacles settle and that growth is slower in more rapid currents. His results did not, however, give an accurate indication of limiting or optimum velocities to which his collectors were exposed.

The experiments which form the subject of the present account are divided into a study of the effects of water currents upon the attachment of cyprid larvae, and into a consideration of the effects of water currents upon the organisms following attachment. Under natural conditions of fluctuating tidal currents and wind drifts it is conceivable that a cyprid might attach during a period of minimum water flow in a particular region and that, once attached, the maximum flow would be insufficient to detach it or to inhibit its growth. The aim of the investigation was to establish the limiting velocities of current for initial and continued attachment and growth.

The advice and suggestions of Dr. Alfred C. Redfield of the Woods Hole Oceanographic Institution are gratefully acknowledged. The writer is also indebted to Mr. Frank L. LaQue of the Development and Research Division, International Nickel Company, Inc. and to Dr. William F. Clapp for permission to include in this paper data from their observations at Kure Beach, N. C. Acknowledgments are also due Mr. D. S. Reynolds, Mr. James Gregg, Mr. Alexander Frue, and Mr. Charles Weiss for their assistance at various times.

METHODS

In order to study the effects of water currents two types of apparatus were designed which would permit the movement of sea-water relative to the experimental surface at predetermined and variable velocities. The first consisted of a rotating disc, immersed in the sea. The second type of apparatus consisted of a series of glass tubes of varying cross-sectional diameter. The rotating disc was employed both in the study of initial attachment of barnacles and in the study of growth subsequent to attachment. The glass tubes of graded diameter were employed in the observations on initial attachment only, as a check against results obtained with the rotating disc.

Water Currents and Attachment on the Rotating Disc

The rotating disc apparatus as used in the experiments here described is essentially similar to a machine described by LaQue (1943) for the purpose of studying the effect of sea-water currents upon corrosion of metal samples, with certain changes in design appropriate to the particular use to which it was put. The modified machine consists of a vertical shaft rotated by means of an electric motor and a system of belts and pulleys. The disc is attached to the shaft by means of a flange and small brass screws and is maintained in a position several inches below low water spring tide. At velocities of rotation used in the experiment no cavitation occurred at this depth.

By changing the arrangement of pulleys the speed of rotation may be varied up to approximately 1750 r.p.m. The rate of movement in knots of any portion of the disc relative to an imaginary stationary body of water is readily calculated as approximately R × D 370, where R is the number of revolutions per minute and D the diameter in inches. This velocity is nominal, however, and does not accurately represent the rate of relative movement between the disc and the water close to its surface. Frictional drag and centrifugal forces produce vortex movements which cause water to flow in a close spiral roughly parallel to the plane of the disc. As a result, the actual flow of water at any part of the disc's surface is less than the nominal rate calculated from the velocity of rotation. Size of the discs used varied up to 28 inches in diameter.

Experimental procedure consisted of bolting a new rotating disc on the shaft and setting the machinery in continuous operation. The disc was removed and examined at suitable intervals. At the same time a similar stationary disc was immersed at a point nearby the rotating disc, at the same depth and resting similarly in a horizontal plane. Observations on the second disc acted as a control for such factors as abundance of organisms, nature of the surface of the disc, etc. The apparatus was used at the edge of a covered slip at the Miami Beach Boat Corporation, where fouling is usually severe. The predominant organism at most times of the year is *Balanus amphitrite niveus* Darwin and this was accordingly selected for observation.

Results

The results of the first rotating disc experiment indicated that the approximate minimum current velocity required to prevent barnacle attachment is 1.1 knots. The disc used was 13 inches in diameter and was rotated at 540 r.p.m. The disc, together with its stationary control, are shown in Figure 1 as they appeared at the end of this experiment. Five barnacles attached to the center of the rotated disc during 23 days but did not grow beyond 2 mm. in greatest width. The limiting velocity was calculated from the ¾ inch diameter of the circle to which the barnacle attachments were restricted (Table I).

TABLE I

Effect of water currents upon attachment of Balanus amphitrite as observed on the lower surface of a horizontal rotating disc

Disc	Date of start	Duration of rotation (Days)	Speed of rotation (r.p.m.)	Maximum diameter at which attachment occurred (Inches)	Maximum nominal velocity at which attachment occurred (Knots)
1 B	8/20/43	23	540	$\frac{3}{4}$	1.1
4 A	11/4/43	16	192	2	1.0
6 A	12/7/43	14	134	$2\frac{1}{2}$	0.9
7 A	1/5/44	19	60	8	1.3
Average	—	—	—	—	1.1

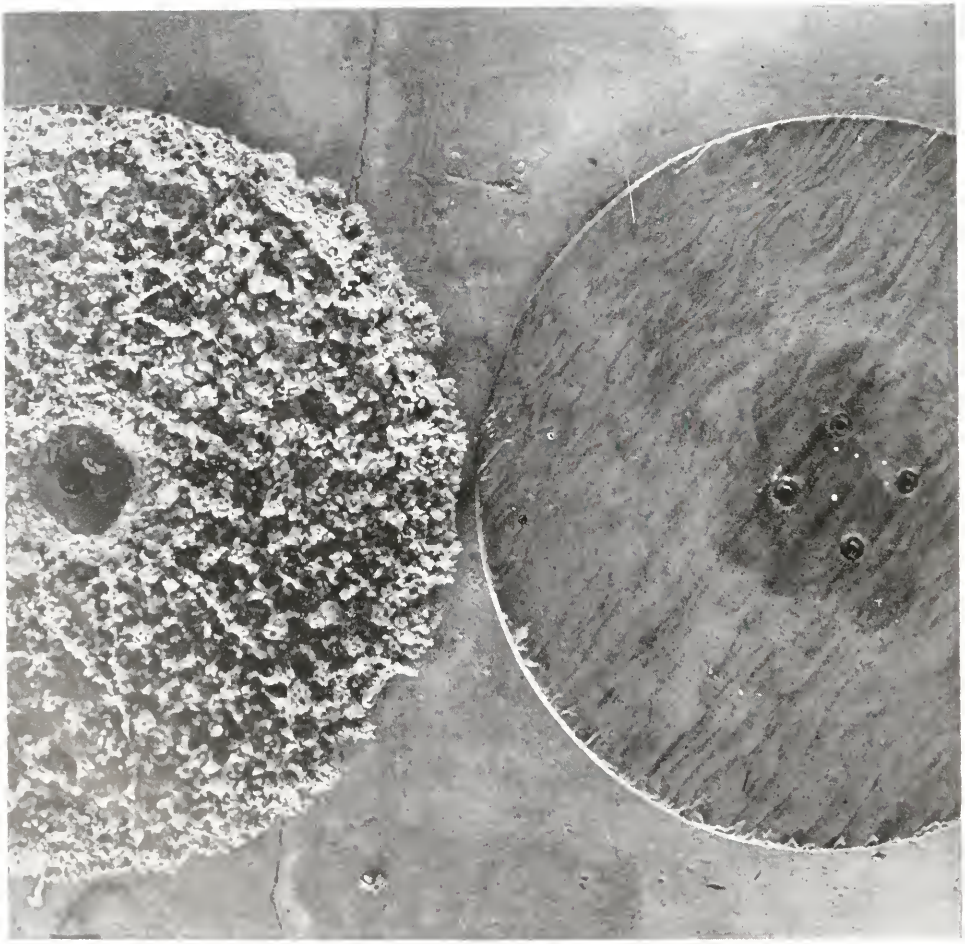

FIGURE 1. Surface of disc rotated at 540 r.p.m. for a period of 23 days, together with stationary control. Diameter of disc, 13 inches.

In order to establish the limiting velocity with greater accuracy the experiment was repeated on three other occasions at slower speeds of rotation, with the results shown in Table I and Figure 2. The nominal velocity limiting barnacle attachment as shown by these experiments varied from 0.9 knots to 1.3 knots with an average of 1.1 knots. In addition to the adverse effect upon barnacle attachment it was noticed that slime film development was greatly reduced on the rotated disc.

ATTACHMENT IN GLASS TUBES

Owing to the difficulty of accurately measuring the rate of water flow along the surface of the rotating disc, an independent method of investigating the relation between water currents and fouling incidence was used. In this case sea water was passed through sections of glass tubing of varying diameter (Fig. 3). Comparison of fouling incidence in each sector was made with the varying linear velocity of the water, calculated on the assumption that linear rate of flow through tubes varies inversely as the square of the diameter of cross-section. In order to estimate errors which might be introduced by virtue of turbulent flow, carmine was introduced into the water during a preliminary test run. It was observed that flow in the main portion of each section was smooth. Areas near to the joints, where turbulence was noted, were not included in the experimental observations of fouling incidence.

The tubes were approximately eight inches long and of 5 cm., 3.7 cm., 2.8 cm., 2.2. cm., and 1.4 cm. internal diameter respectively and were joined by means of fitted rubber stoppers. Attachment of barnacle larvae was encouraged by excluding direct sunlight. The water flow was provided by means of a centrifugal pump, with the experimental tubes arranged on the inlet side in order to avoid possible mechanical damage to the larvae.

While the apparatus was in operation the rate of flow was checked daily by volumetric measurement at the outlet. At the conclusion of each test run the tubes were carefully examined for signs of fouling.

Results

Observations on the glass tubes showed that limiting velocities for attachment of *Balanus amphitrite* were between 0.5 and 1.0 knot. During the first experiment with this apparatus, sea water was run through it at a rate between 13 and 16 liters per minute, on a catwalk extending over the water at the University of Miami Marine Laboratory. Fluctuations in the height of tide and consequently the suction head of the seawater pump gave rise to this fluctuation in current velocity. Barnacles attached in the two larger tubes within ten days after beginning the experiment. From the calculated linear velocity of the narrower of these tubes it appeared that barnacles are not prevented from attachment by currents varying between 0.5 and 0.6 knot (Table II). In the next smaller tube where no barnacles attached the rate of flow varied from 0.8 to 1.0 knot. The limiting velocity therefore lies between 0.5 knot and 1.0 knot. The appearance of the tubes at the end of a similar experiment is illustrated in Figures 3 and 4. The upper figure of the limits is lower than the critical velocity as measured on the rotating disc. However, in view of the difference in hydrodynamical conditions involved in the two methods, which was discussed previously, the results appear to be reasonably consistent.

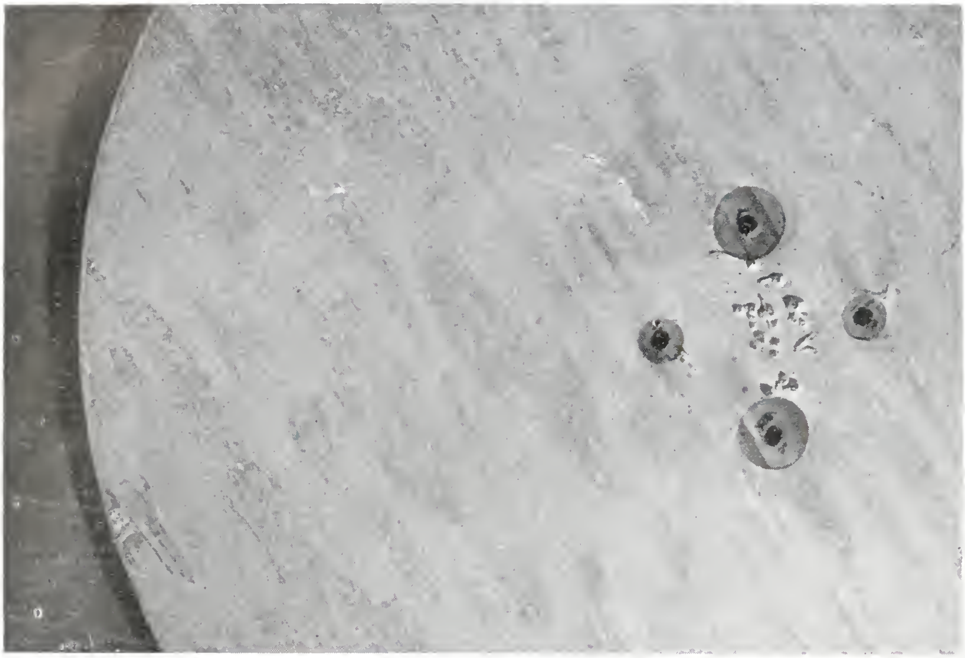

FIGURE 2. Portion of disc rotated at 192 r.p.m. for a period of 16 days, showing barnacles attached at center. Diameter of disc, 24 inches.

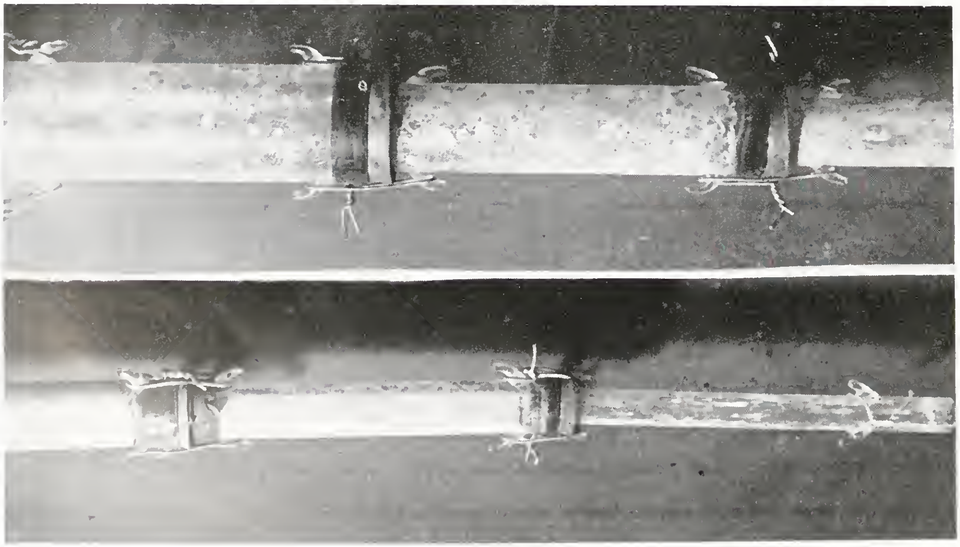

FIGURE 3. Series of five tubes of increasing cross-sectional diameter. Shows development of barnacles in wider tubes only, following prolonged passage of sea-water.

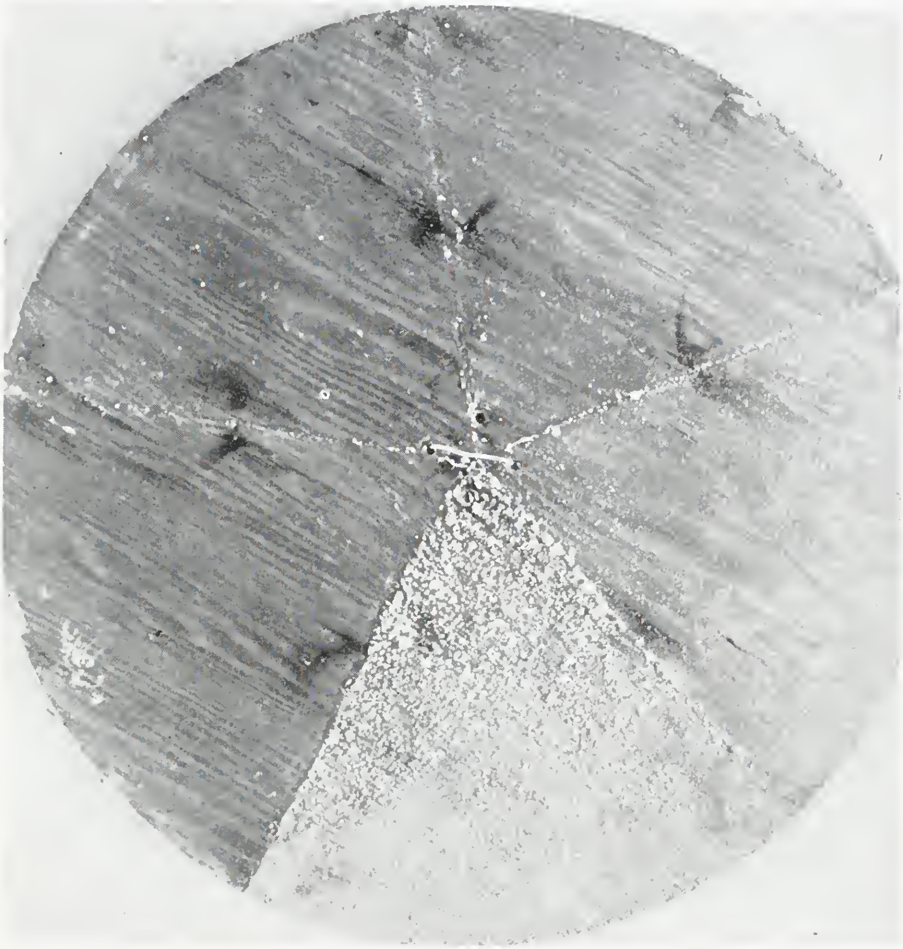

FIGURE 4. Surface of disc following 16 days of stationary immersion beginning January 8, 1944. Individual sectors of the disc were exposed for periods of 6 hrs., 1 day, 2 days, 5 days and 16 days. Diameter of disc, 28 inches.

At a later date the glass tubes were exposed at Kure Beach, N. C., by Mr. F. L. LaQue and Dr. W. Clapp, under conditions allowing a more constant flow of sea water. By reducing the flow of water in successive experiments the critical velocity for attachment was confined more closely between 1.1 and 1.3 knots for *Balanus improvisus* and below 0.8 knot for *B. eburneus*. These results are included in Table II with the permission of LaQue and Clapp.

Confirmatory results have been reported by Turner (1945) from an experiment recently conducted at Kure Beach in which a system of steel pipes of graded diameter was exposed to sea water circulation for 98 days. Fouling with mussels, barnacles, and tube worms occurred where the current velocity was one knot or less but not where it was 1.8 knots or greater.

TABLE II

Attachment of Balanus species upon vertical glass tubes of graded diameter during the passage of sea-water

Period and place	Rate of flow. Liters per minute	Equivalent velocity in glass tubes (Knots)				
		1.4 cm. diameter	2.2 cm. diameter	2.8 cm. diameter	3.7 cm. diameter	5.0 cm. diameter
11/17/43 to 11/27/43 Miami Beach	13–16	3.0–3.7 —	1.2–1.6 —	0.8–1.0 —	0.5–0.6 *B. amphitrite*	0.2–0.3 *B. amphitrite*
5/20/44 to 7/12/44 Kure Beach*	21	5.3 —	2.3 —	1.4 —	0.8 Balanus sp.	0.4 Balanus sp.
7/12/44 to 9/18/44 Kure Beach*	12.7	3.2 —	1.3 —	0.8 Balanus sp.	0.5 Balanus sp.	0.2 Balanus sp.
9/18/44 to 11/25/44 Kure Beach*	10.8	2.7 —	1.1 *B. improvisus*	0.7 *B. improvisus*	0.4 *B. eburneus* *B. improvisus*	0.2 *B. eburneus* *B. improvisus*

* Observations by W. F. Clapp and F. L. LaQue.

GROWTH ON THE ROTATING DISC

For the purpose of studying the effect of water currents upon barnacles already attached the rotating disc procedure was modified. Both rotating and stationary discs were allowed a preliminary period of stationary immersion during which barnacles became attached. Equal sectors of the disc were temporarily protected by means of cloth attached with thumb tacks. The cloth was removed from each sector after increasing intervals of time with the result that at the end of the preliminary period of immersion the maximum age of the organisms attached to the different parts of the disc varied from 16 days to 6 hours. The barnacles on each sector were then counted and the maximum diameter of the largest barnacle in each sector was measured.

Following the period of stationary immersion the control disc was allowed to remain in a stationary position and the other was placed on the rotating shaft for a further period. Both discs were examined and the number of barnacles per square inch and their greatest diameter were recorded separately for each sector and for areas at successive distances from the center of the disc.

Results

The first experiment of this series provided an initial 16 days' period of stationary immersion during which both experimental and control discs became seeded with barnacles. At the end of this period barnacles had been attached on the five sectors for maximum periods of 6 hours, 1 day, 2 days, 5 days, and 16 days respectively (Fig. 4). Immediately following the period of stationary growth, the experimental disc was rotated at an angular velocity of 60 r.p.m. for 19 consecutive days, while the control disc was allowed to remain in a stationary position for the same period. The appearance of both discs at the end of the experiment is shown in Figures 5 and 6.

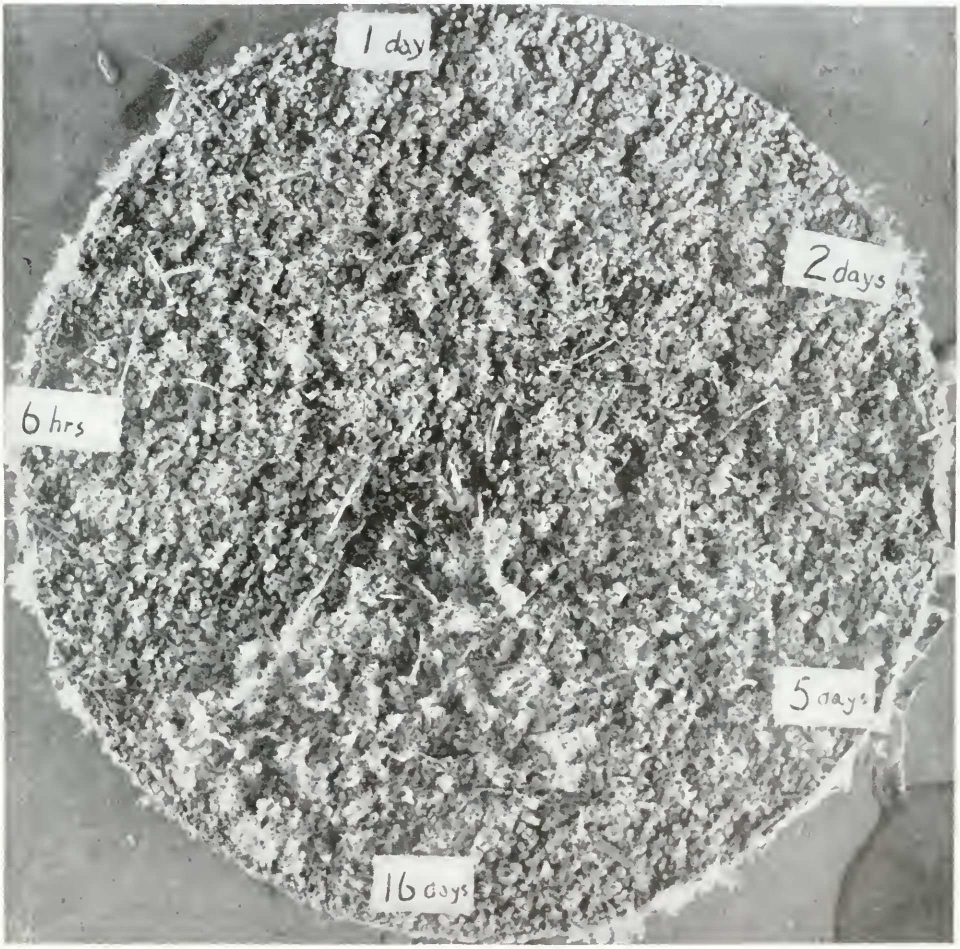

FIGURE 5. Same disc as in Figure 4 after further period of 19 days stationary immersion. Control to disc shown in Figure 6.

Examination of the discs at the conclusion of the test showed that while increasing current velocities diminished the growth rate and finally brought about loss of attachment, the weaker currents appeared to enhance growth. Thus, when rotated on Sector I, where growth had continued for 6 hours before rotation, *B. amphitrite*

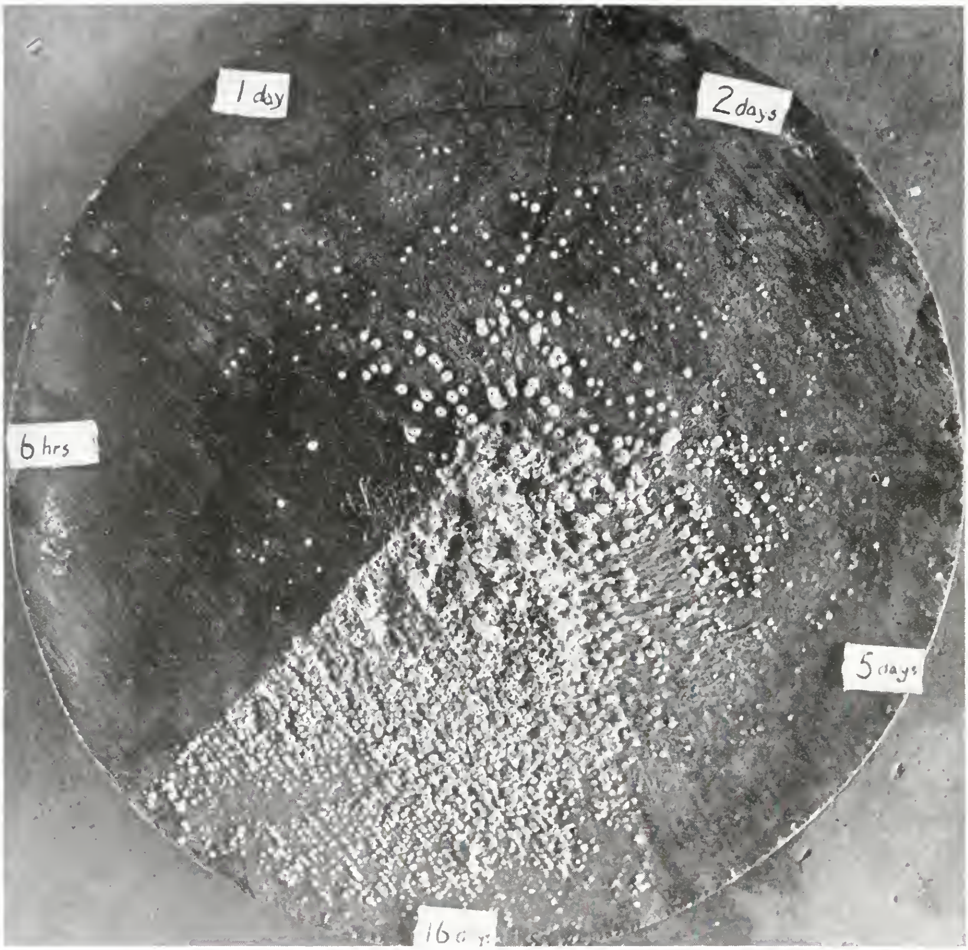

FIGURE 6. Similar disc to that of Figure 4 after period of 19 days rotation at 60 r.p.m. Age of barnacles before rotation is marked on each sector.

neither continued development nor remained attached at diameters beyond 16 inches, corresponding to a current of 2.7 knots (Table III). Within Sector VI, however, with barnacles originally 16 days old, a small number remained attached even at the outer edge of the disc, where the water flow approximated 4.7 knots. On the intervening sectors with barnacles of intermediate ages, the current velocities bringing about complete loss of attachment showed values between these extremes. Probably because of further attachments on the stationary control, the density of attachments on all sectors of the rotated disc was below that of the control.

The effects upon growth rate are illustrated by the observations in Table IV. On Sector I, within a 4 inch diameter, equivalent to 0.7 knot, barnacles reached a maximum width of 8 mm., compared with growth on the control of only 7 mm. At 2.3 knots, growth was reduced to only 3 mm. and at 2.7 knots, no barnacles re-

TABLE III

Effect of water currents upon adhesion of barnacles of different ages when subjected to 19 days rotation upon a submerged disc, as shown by density of attachment in number per square inch. Experiment initiated January 8, 1944

Position on disc		Age before rotation				
Diameter (Inches)	Nominal water velocity (Knots)	Sector I (6 hours)	Sector II (1 day)	Sector III (2 days)	Sector IV (5 days)	Sector V (16 days)
0–28*	0	Dense	Dense	Dense	Dense	Dense
4	0.7	1.7	1.7	1.7	2.0	Too crowded to count
6	1.0	0	0.4	0.9	1.7	
8	1.3	0	0.4	1.0	1.3	
10	1.7	0.1	0.4	1.0	1.0	
12	2.0	0.2	0.4	0.6	1.9	
14	2.3	0.1	0.4	0.9	1.3	
16	2.7	0	0.4	0.6	0.9	
18	3.0	0	0.1	0.2	0.6	
20	3.3	0	0	0.1	0.4	
22	4.0	0	0	0	0	
28	4.7	Few in cracks	0	Few in cracks	Few in cracks	Few

* Stationary control.

mained on the sector. At intermediate current velocities conditions of growth lay between these two extremes, with normal growth occurring between 0.7 and 1.7 knots.

Similar observations were made upon barnacles of greater initial age within the remaining sectors. Thus, on Sector IV, where development had continued for a maximum period of 5 days before rotation, barnacles showed enhanced growth rate in currents up to one knot, normal growth similar to that of the control up to 2 knots, and almost complete cessation of growth at 3.3 knots. No barnacles remained on this sector at 4 knots.

Similar conditions of enhanced growth at low velocities, normal growth at intermediate velocities and cessation of growth with loss of attachment at higher velocities were observed on the remaining sectors, with barnacles of different initial ages.

The anomalous absence of enhanced growth on Sector V is probably due to the crowded condition of the barnacles which had become attached during a 16 day stationary period. The one day old barnacles were apparently less affected by the

TABLE IV

Effect of water currents upon growth of barnacles of different ages when subjected to 19 days rotation upon a submerged disc, as shown by diameter of largest barnacle in millimeters. Experiment initiated January 8, 1944

Position on disc		Age and size before rotation				
Diameter (Inches)	Nominal water velocity (Knots)	Sector I 6 hours (<1 mm.)	Sector II 1 day (<1 mm.)	Sector III 2 days (<1 mm.)	Sector IV 5 days (2 mm.)	Sector V 16 days (5 mm.)
0–28*	0	7 mm.	7 mm.	8 mm.	8 mm.	9 mm.
4	0.7	8 mm.	8 mm.	8 mm.	9 mm.	None more than 8 mm.
6	1.0	—	8 mm.	9 mm.	9 mm.	
8	1.3	—	8 mm.	8 mm.	8 mm.	
10	1.7	6 mm.	7 mm.	6 mm.	8 mm.	
12	2.0	5 mm.	5 mm.	6 mm.	8 mm.	
14	2.3	3 mm.	6 mm.	4 mm.	6 mm.	
16	2.7	—	5 mm.	3 mm.	5 mm.	
18	3.0	—	1.5 mm.	1.5 mm.	4 mm.	7 mm.
20	3.3	—	—	1.5 mm.	2.5 mm.	6 mm.
24	4.0	—	—	—	—	6 mm.
28	4.7	1.5 mm.	—	1.5 mm.	4 mm.	5 mm.

* Stationary control.

inhibitory action of water currents than the two day old barnacles. Otherwise, the adverse effects of water currents increased with their velocity, and decreased with the initial age of the barnacles.

On all sectors a few barnacles continued to grow at the perimeter of the disc where the presence of cracks provided local flow pockets with relatively still water.

The first experiment demonstrated an actual loss of barnacles at current velocities having critical values for the different initial ages of barnacles. In order to determine the time required for the loss of attachment to occur, a second experiment was carried out in which the disc was examined, not only at the beginning and end of the rotation period, but also at intervening times. The density of attachment and maximum size of barnacles were observed at each examination, as in the previous experiment.

The experiment was carried out during a period of heavy barnacle set. At the end of the stationary period, extending from February 19 to March 1, 1945, the density of attachments upon each sector, and particularly upon the sectors longest exposed, was very much greater than in the previous experiment.

TABLE V

Density of barnacle attachment compared with rate of flow of water relative to the surface of a rotating disc, expressed in number per square inch *
(*Figures in brackets indicate density upon a stationary panel serving as control;* D, *over 75 per square inch*)

Stationary growth period	Diameter of disc (Inches)	Velocity (Knots)	Period of rotation							
			0 hrs.	1 hr.	12 hrs.	3 days	5 days	7 days	9 days	11 days
Sector I	Control	(0)	(30)	(30)	(30)	(30)	(30)	(60)	(D)	(D)
	4	1.5	30	25	25	20	15	10	8	5
	8	3	30	30	20	20	15	10	8	6
6 hrs.	12	5	30	30	20	20	15	6	6	6
	16	6.5	30	30	20	20	15	6	6	5
	20	8	30	30	20	20	15	4	4	2
	24	10	35	30	20	20	12	4	4	2
Sector II	Control	(0)	(30)	(30)	(30)	(30)	(30)	(60)	(D)	(D)
	4	1.5	30	30	30	30	20	15	15	8
	8	3	30	30	30	30	20	15	15	12
12 hrs.	12	5	30	30	30	30	20	15	15	6
	16	1.5	30	30	30	30	20	15	15	6
	20	8	30	30	30	30	20	10	10	5
	24	10	30	30	30	10	7	7	7	4
Sector III	Control	(0)	(30)	(30)	(30)	(30)	(30)	(60)	(D)	(D)
	4	1.5	30	30	30	20	20	12	12	10
	8	3	30	30	30	20	20	12	12	12
1 day	12	5	30	30	30	20	12	10	10	10
	16	6.5	30	30	30	20	12	10	10	6
	20	8	30	30	30	20	12	7	7	5
	24	10	30	30	30	7	5	5	4	4
Sector IV	Control	(0)	(30)	(30)	(30)	(40)	(40)	(50)	(D)	(D)
	4	1.5	30	30	30	20	20	17	17	15
	8	3	30	30	30	24	20	20	20	19
2 days	12	5	30	30	30	24	20	20	15	9
	16	6.5	30	30	30	24	20	20	15	8
	20	8	30	30	30	15	10	10	10	7
	24	10	30	30	30	10	7	7	6	6
Sector V	Control	(0)	(30)	(30)	(30)	(50)	(50)	(D)	(D)	(D)
	4	1.5	35	35	35	32	32	32	30	30
	8	3	35	35	30	30	30	30	30	30
	12	5	35	35	30	30	30	30	30	24
5 days	16	6.5	35	35	30	30	30	30	30	15
	20	8	35	35	30	30	30	30	30	15
	24	10	35	35	25	25	25	25	25	15
Sector VI	Control	(0)	(70)	(70)	(70)	(70)	(D)	(D)	(D)	(D)
	4	1.5	75	75	75	75	75	75	75	50
	8	3	75	75	75	70	70	70	70	30
10 days	12	5	75	75	75	70	70	70	70	30
	16	6.5	75	75	75	70	70	70	70	25
	20	8	75	75	75	70	70	70	70	20
	24	10	75	75	75	70	70	70	70	20

* Experiment initiated 2/19/45.

Observations showed that, as in the previous experiment, losses in attached barnacles occurred roughly in proportion to the current velocity, although at no point of the disc was the entire set lost. Similarly, the limiting rate of flow necessary for inhibition of growth or for loss of attachment increased generally with the initial age of the barnacles. Since observations were made at intervals during the experiment it was also possible to note the period of rotation necessary to bring about the first losses in attachment. This also increased generally with increasing age of initial growth. The observations are set forth in detail in Tables V and VI and summarized in Table VII.

In Table V the number of barnacles per square inch is recorded for various distances from the center of the disc, and for each individual sector. These observations are repeated for various intervals of time during the experiment.

An increase took place in numbers on the control panels due to continued attachment of cyprids, whereas on the rotated disc barnacles became progressively reduced in number as the experiment continued. After one hour of rotation the only losses were among the six hour old barnacles on Sector I. These losses were very small, however, and although observed at current velocities of 1.5 knots and 10 knots, they did not occur at intervening velocities. The first significant losses took place twelve hours after the start of rotation among barnacles less than twelve hours old. Of the older ones, only those 5 days old showed losses, amounting to a change at most velocities from 35 to 30 per unit area.

Barnacles on the remaining sectors, varying in age from twelve hours upwards, were not observed to diminish in number until the third day of rotation. The smallest loss over this period took place among the twelve hour barnacles, which were only lost at speeds of ten knots, whereas, after a similar period of rotation, other barnacles of greater ages had been lost even at 1.5 knot current velocity. Ten day old barnacles were not greatly disturbed by 1.5 knot currents until after eleven days' rotation. These observations, summarized in Table VII, indicate that in general the velocity of current necessary to dislodge barnacles increased with the age they had reached before the experiment. Barnacles twelve hours old, however, showed a marked increase in resistance to being dislodged, even compared to those ten days old, whereas those two days old, on the other hand, seemed to be less resistant than younger or older ones. It is also noteworthy that in general the duration of rotation necessary to bring about the first losses depends much more upon the initial age of the barnacle than upon the current velocities.

In a similar manner the percentage of the original number of barnacles remaining following an eleven day period of rotation increased with increasing age before rotation. The ten day old barnacles at the end of the experiment, however, showed poorer resistance and had a lower percentage of continued attachment than in the case of the five day barnacles. It must be noted, however, that the ten day barnacles were densely crowded and it is possible that this factor decreased their resistance to dislodgement.

Further examination of the results in Table V discloses that losses continued to occur to some extent on all sectors at the end of eleven days, although they were less on those sectors where initial age of the barnacle was greater.

Observations of the size of barnacles recorded during the experiment are given in Table VI. On Sector I the six hour old barnacles within a diameter of 8 inches, equivalent to a three knot water current, continued to grow, although at a slow rate.

TABLE VI

*Growth of barnacles in relation to flow of water over the surface of a rotating disc, expressed as greatest diameter in millimeters **
(Figures in brackets refer to stationary control)

Stationary growth period	Diameter of disc (Inches)	Velocity (Knots)	Period of rotation							
			0 hrs.	1 hr.	12 hrs.	3 days	5 days	7 days	9 days	11 days
Sector I	Control	(0)	(0.3)	(0.3)	(0.3)	(0.5)	(0.8)	(1.3)	(2.5)	(3.0)
	4	1.5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5
	8	3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
6 hrs.	12	5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	16	6.5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	20	8	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	24	10	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Sector II	Control	(0)	(0.3)	(0.3)	(0.3)	(0.6)	(0.8)	(2.5)	(3.0)	(3.5)
	4	1.5	0.3	0.3	0.3	0.3	0.3	0.3	1.0	1.5
	8	3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
12 hrs.	12	5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	16	6.5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	20	8	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	24	10	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Sector III	Control	(0)	(0.3)	(0.3)	(0.3)	(0.6)	(0.8)	(2.0)	(3.0)	(3.5)
	4	1.5	0.3	0.3	0.3	0.5	0.5	1.0	2.0	3.0
	8	3	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
1 day	12	5	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	16	6.5	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	20	8	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	24	10	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
Sector IV	Control	(0)	(0.3)	(0.3)	(0.5)	(0.8)	(1.0)	(3.0)	(3.5)	(4.0)
	4	1.5	0.3	0.3	0.5	1.0	1.0	1.3	1.3	1.5
	8	3	0.3	0.3	0.3	0.8	0.8	0.8	0.8	1.0
2 days	12	5	0.3	0.3	0.3	0.8	0.8	0.8	0.8	1.0
	16	6.5	0.3	0.3	0.3	0.3	0.8	0.8	0.8	0.8
	20	8	0.3	0.3	0.3	0.3	0.8	0.8	0.8	0.8
	24	10	0.3	0.3	0.3	0.3	0.8	0.8	0.8	0.8
Sector V	Control	(0)	(0.8)	(1.0)	(1.5)	(2.5)	(3.0)	(4.0)	(5.0)	(6.0)
	4	1.5	0.5	0.8	1.0	2.5	2.5	3.0	3.0	3.5
	8	3	0.5	0.8	0.8	1.5	1.5	1.5	1.5	1.5
5 days	12	5	0.5	0.5	0.8	1.5	1.5	1.0	1.5	1.5
	16	6.5	0.5	0.5	0.8	1.5	1.5	1.0	1.5	1.5
	20	8	0.5	0.5	0.8	1.5	1.5	1.0	1.5	1.5
	24	10	0.5	0.5	0.8	0.8	0.8	0.8	0.8	0.8
Sector VI	Control	(0)	(2.5)	(2.0)	(2.5)	(4.0)	(5.0)	(7.0)	(9.0)	(9.0)
	4	1.5	2.0	2.0	2.0	3.5	3.5	4.5	4.5	5.0
	8	3	2.0	2.0	2.5	3.0	3.0	3.5	3.5	3.5
10 days	12	5	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5
	16	6.5	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5
	20	8	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5
	24	10	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5

* Experiment initiated 2/19/45.

TABLE VII

*Summary of observations on the effect of water currents produced by rotation upon attached barnacles. Expressed as multiples of normal growth, period before loss in number first occurred, and percentage remaining at the end of eleven days **

Current velocity (Knots)	Period of stationary growth					
	6 Hrs.	12 Hrs.	1 Day	2 Days	5 Days	10 Days
1.5	0.1 × (1 Hour) 20%	0.4 × 5 Days 30%	0.8 × 3 Days 30%	0.3 × 3 Days 50%	0.6 × 3 Days 90%	0.6 × 11 Days 70%
3	*Inhibition* 12 Hours 30%	*Inhibition* 5 Days 40%	*Inhibition* 3 Days 40%	0.3 × 3 Days 60%	0.2 × 2 Days 90%	0.3 × 3 Days 40%
5	— 12 Hours 20%	— 5 Days 20%	— 3 Days 30%	0.1 × 3 Days 30%	0.2 × 2 Days 70%	0.3 × 3 Days 40%
6.5	— 12 Hours 20%	— 5 Days 20%	— 3 Days 20%	*Inhibition* 3 Days 30%	0.2 × 2 Days 60%	0.3 × 3 Days 30%
8	— 12 Hours 20%	— 5 Days 20%	— 3 Days 20%	— 3 Days 20%	*Inhibition* 2 Days 60%	0.3 × 3 Days 30%
10	— (1 Hour) 10%	— 3 Days 20%	— 3 Days 20%	— 3 Days 20%	— 2 Days 60%	0.3 × 3 Days 30%

* Experiment initiated 2/19/45.

Even at velocities below 1.5 knots the growth was considerably less than that of the stationary control. The ten day old barnacles on Sector VI continued to grow near the circumference, although subjected to ten knot currents. Barnacles of intermediate age showed a reduction of growth inversely dependent upon their initial age, and directly dependent upon the current. Barnacles less than two days old ceased growth at water currents over three knots, and only the ten day old barnacles continued growth at ten knots.

At the end of this experiment the remaining barnacles were examined to determine whether any considerable portion remaining attached was dead. A few of the older barnacles had lost their soft parts, but it was difficult to obtain a reliable criterion as to the condition of the remainder. It was not definitely established, therefore, what proportion had died.

Differences between the results of this and the previous experiment lie mainly in the higher current velocities required to bring about growth inhibition and loss of attachment and the absence of complete loss at any part of the disc in the later experiment. This apparent anomaly may be partly due to the much greater density of initial attachment, which may have reduced the *effective* velocity of the currents by providing a much more irregular surface. It is also conceivable that had the experiment continued for the full 19 day period of the first experiment rather than 11 days only, the growth inhibition effect might have appeared at lower velocities and might have resulted in complete loss at some parts of the disc.

Discussion

The experimental results indicated that current velocities limiting the attachment of barnacles lie in the neighborhood of one knot and that the limits for the three species, *Balanus eburneus, B. amphitrite,* and *B. improvisus* are in ascending order of magnitude. That a one knot current should limit attachment is in accordance with the conclusions of Visscher and earlier workers that attachment of barnacles does not readily take place upon ships in motion.

It further appears that growth rates of previously attached barnacles are reduced by water currents in inverse proportion to the age of the barnacle and directly in proportion to the current velocity. The effect of crowding together is further to reduce the growth rate. The effect of very low current velocities is to increase the rate of growth. A slight loss of attachment occurs at all current velocities, but this may be partly due to overcrowding. Large and significant losses take place at velocities which are sufficient to reduce the growth rate. These losses begin some hours after the start of rotation, and continue for a period of at least eleven days.

Since losses due to dislodgement do not occur during the first few hours of rotation, except in the case of barnacles attached within six hours or less, and since they continue to take place during the entire period of rotation, it seems probable that they are not directly due to mechanical action. Possibly the mechanism is one of interference with feeding processes, followed by reduced growth rate, death and diminished adhesion.

A further point arising out of the experimental data is the fact that at equivalent current velocities 12 hour and 24 hour old barnacles show greater growth and less loss of attachment than those somewhat older. It is possible that at this stage of development, with metamorphosis incomplete, an orientation to the current may occur which facilitates feeding, although this was not actually observed.

Once attached the barnacles are able to withstand current velocities of increasing magnitude, although for the first two or three weeks the growth rate is considerably reduced. Loss of attachment appears to follow reduced growth rate and may involve actual death from lack of food. The current velocities required to bring this about are sufficiently low to provide support for Visscher's conclusion that barnacles are killed by ocean passages of 500 miles or more, if vessels of more than 10 knots performance are considered, or if the age of attached barnacles is no greater than a 10 day stay in port would allow. On the other hand, current velocities lying below this figure, such as would be produced by slow sailing vessels, would appear from the experimental data to be insufficient to bring about a loss of the attached barnacles. In the case of faster ships, stays in port of longer than a few days might also permit the accumulated barnacles to survive an ocean passage. This type of ship service would readily provide a means of distribution for barnacles. It would explain Fischer-Piette's observations of the appearance of *Balanus amphitrite* on Mediterranian shores, and would support his hypothesis of distribution by ships.

The enhanced growth rate in current velocities of 1.3 knots and less, depending on the age of the barnacle, provides a quantitative expression for the observations of Rice, Moore, Kitching, and others upon the increased growth of barnacles subjected to water currents.

The conditions in a harbor or an embayed or open coast are rarely such that tidal currents in excess of one knot are continuously present. Under these conditions, therefore, there would be no obstacle to the attachment of barnacles. Once attached, however, the barnacles would usually be subjected to intermittent tidal currents.

No data are available as to the effects of intermittent currents but it would be reasonable to assume that these would be, if anything, less than the effects of continuous currents. The data presented here would therefore make it appear unlikely that any but the strongest of tidal currents would prevent the attachment and continued development of barnacles on rocks or stone, and wooden piling. Where fairly continuous currents of less than 1.5 knots were present the growth would be encouraged.

A different situation occurs in estuaries where continuous currents might occur. Here, however, the limiting factor, according to the work of previous authors, is probably lowered salinity, since continuous currents would scarcely permit of the estuary remaining salt.

Where wave action is strong the effects might be considered as equivalent to strong currents of frequently changing direction, which might conceivably inhibit attachment or growth, even below tide levels. Even where attachment occurred during temporary lulls, the strong recurrent wave action might be expected to dislodge the barnacles. This may explain the observations of Stephenson and his co-workers that some species of barnacles do not occur in a zone of heavy wave action.

The effect of water currents under natural conditions may therefore be summarized as one of enhanced growth with velocities below 1.5 knots, and one of reduced growth above this. It would rarely happen under natural conditions that water currents alone would prevent barnacle colonization. Strong wave action, however, might be expected to have this effect.

Certain qualifications should be added to the above discussion which may explain some anomalous observations. The arguments above are based on the calculated rates of flow of water over the rotating disc, and the results of experiments have indicated that these rates are slightly higher than the actual rates of flow taking place. The error, however, does not appear to be greater than 10 per cent to 20 per cent, and does not qualitatively affect the validity of the general conclusions.

Since the observations were made upon the relatively smooth surface of a wooden disc, they would not be applicable to very rough surfaces where local pockets of relatively still water might develop, to the areas immediately adjacent to lapped plates of ships' hulls, or to portions of piling or other submerged surfaces where the contours and configuration might produce local stagnation effects.

SUMMARY

1. The work of previous authors, dealing with the effect of water currents upon barnacle attachment, growth and distribution is briefly reviewed.

2. Experiments were conducted to determine the effects of water currents upon the attachment and growth of barnacles, and particularly of *Balanus amphitrite.*

Submerged rotating discs and glass tubes of graded cross-sectional diameter were employed to provide variations in current velocity.

3. The velocity of water current limiting attachment appears to lie between 0.5 and 0.9 knot for *Balanus amphitrite,* between 0.4 and 0.7 knot for *B. eburneus,* and above 1.1 knots for *B. improvisus.*

4. Following attachment the growth rate of barnacles was found to be increased by water currents of velocity less than 1.5 knots and to be decreased by currents with velocities in excess of this. The adverse effects of water currents were found to decrease with increasing age of the barnacles subsequent to attachment. Six hours after attachment, growth rate was reduced to one-third of normal by a 1.5 knot current and completely stopped by a 3 knot current. Five days after attachment, growth was prevented by currents ranging between 3.3 and 8 knots.

Loss of attachment appeared to some extent among all barnacles in which growth rate was reduced. This loss was greatest at velocities bringing about complete cessation of growth.

5. It is suggested that loss of attachment is due to interference with the feeding process, followed by reduction of growth rate, death, and diminished adherence. Possibly due to an orientation to the current which facilitates feeding, barnacles attached for one day or less show less retardation of growth rate and loss of adherence than barnacles two days old.

6. Since tidal currents are almost invariably intermittent it appears from the data presented that they are not sufficient to prevent the colonization of suitable surfaces by barnacles, except where the velocities are unusually high.

It also follows from the numerical results obtained that on vessels making short stays in port and relatively long voyages, little permanent barnacle fouling will occur, since those organisms which attach will be killed and at least a portion of them dislodged. The evidence does not preclude, however, the continued growth of barnacles upon slow vessels making longer stays in port, and their geographical distribution by this means.

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MICRURGICAL STUDIES ON CHIRONOMUS SALIVARY GLAND CHROMOSOMES [1]

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Particular interest in the giant salivary gland chromosomes of dipteran larvae was awakened by Painter (1933) and Heitz and Bauer (1933) who demonstrated the existence of a close relationship between the genetic significance of the chromosomes and their structure. Regarding the gross structure of these chromosomes there is general agreement, but not for fine details. Bauer (1936) and Painter and Griffen (1937) offered cytological evidence for a polytene concept of chromosome structure from observation, in fixed and stained preparations, of numerous longitudinal striations which they considered to be chromonemata. Metz and Lawrence (1937) and Metz (1941) maintained that the longitudinal striations appearing in these fixed and stained preparations were merely the result of drawing out the walls of alveoli. Buck (1942) afforded support for this view by showing in osmic vapor-treated chromosomes that an original alveolar structure could be reversibly transformed into a fibrillar-like one by micromanipulation. Kodani (1942), using cytolytic methods with alkali and urea, found evidence for only four chromonemata in each chromosome, a condition which Painter and Griffen (1937) and Buck (1937) observed in the early larval stages, but not in the giant chromosomes of later stages. More recently, Frolova (1944) has given experimental support to the polytene concept by demonstrating the presence of numerous longitudinal strands in fixed chromosomes after partial digestion with nuclease preparations.

Aside from their cytogenetic interest, the salivary gland chromosomes, because of their large size and visibility in vivo, make especially suitable material for studies on physical properties. Using the micrurgical technique, various investigators (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; and Pfeiffer, 1940) have found the freshly isolated salivary gland chromosomes to be tough, viscid, elastic gels. These observations are in essential agreement with those first made by Chambers and Sands (1923) on the chromosomes of Tradescentia pollen mother cells and by Chambers (1924a) on those of Dissosteria spermatocytes. Buck (1942), using osmic vapor-treated chromosomes, reported more detailed findings on elasticity. That the physical properties of the chromosomes may be easily modified by the presence of torn cytoplasm or calcium ions was shown by Duryee (1941) in his studies on amphibian chromosomes. The observations of Chambers (1924b) that torn cytoplasm is acidic, and of Chambers and Reznikoff (1926) that the micro-injection of various salts into protoplasm may have detrimental effects, further point out the need for precaution in studying the physical properties and structure of the chromosomes.

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[1] This work, done at Washington Square College, New York University, and at the Marine Biological Laboratory, Woods Hole, Mass., is presented in partial fulfilment of the requirements for the degree of doctor of philosophy at New York University.

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This investigation is concerned with the structure and physical properties of the salivary gland chromosomes as observed within the intact fresh cell and after isolation into media designed to maintain them in the fresh condition.

Grateful acknowledgment is made to Prof. Robert Chambers, under whose guidance this work was done, for his constant aid and invaluable criticism.

Materials and Methods

The species selected for study were *Chironomus plumosus, C. tentans,* and certain smaller unidentified species of Chironomus. Chironomus larvae were chosen in preference to those of other Diptera because of their larger chromosomes (maximum size, 20 μ in diameter and 150 μ long) and the flattened shape of the salivary gland, factors which facilitate micromanipulation. Moreover, the chromosomes were always plainly visible in the living cell, the bands being especially well defined. Incidental observations were made on Drosophila chromosomes and they were found to agree in all important respects.

The glands were carefully removed from mature larvae under a dissecting microscope and immediately immersed in either a drop of Chironomus hemolymph or amphibian Ringer's solution which was found to be isotonic. Care was taken to avoid the presence of any tissue but the gland itself since otherwise the medium became unduly acid. The hemolymph was obtained by inserting a pipette through the body wall of a larva into the hemocoele. In order to be satisfactory the hemolymph had to be freshly obtained and immediately covered with paraffin oil to prevent the development of alkalinity which occurred on exposure to air.

The micromanipulation was carried out immediately after mounting a single intact gland in the medium on a thoroughly cleaned coverslip which was then inverted over the moist chamber of a Chambers micromanipulator. Structural changes within the chromosomes of the intact cells do not appear for many hours in hemolymph or Ringer's solution, but extra precautions were taken by making observations within 15 minutes after mounting the glands. The micropipettes routinely used had an inside bore of never over one μ, and the microneedles used for fine dissection of the chromosomes tapered rapidly to points approaching the limit of visibility.

Nuclei and chromosomes were removed from disintegrating cytoplasm as quickly as possible. This was necessary in order to preserve their normal appearance, an observation in agreement with that of Duryee (1937) on amphibian germinal vesicles. To remove the chromosomes, an entire gland was mounted under a dissecting microscope in a medium, to be described later, in which the chromosomes were to be isolated. By means of fine steel needles a cell was quickly torn and the nucleus ruptured. The torn nucleus remained in situ, and, by exerting a gentle pressure on the torn cell, the chromosomes could be forced out of the nucleus and floated away without coming in contact with the injured cytoplasm. The loosely adhering clump of chromosomes was immediately transferred by means of a lip pipette to a fresh drop of the medium to be used, then mounted in the usual manner in the moist chamber. In this way the chromosomes were found to retain most nearly their normal appearance and physical properties after isolation, their subsequent condition depending on the medium in which they remained. No medium was found which would maintain the chromosomes in their normal condition indefinitely nor were extensive attempts made to find such a medium. Among the media tried and found to be unsatisfactory were hemolymph, Ringer's solution, paraffin oil, 0.1 M NaCl–0.01 M KCl solution used by Duryee (1941), 0.25 per cent egg albumen solution used by Melland (1938), and 0.3 M sucrose solution suggested by the work of Chambers and Sands (1933). These special solutions were used without regard to pH conditions.

From determinations on a great variety of cells including the Chironomus salivary gland nucleus, Chambers (1929) found the intranuclear pH to be within 7.6–7.8. It would be expected therefore that the medium in which the isolation of the chromosomes is to be done should have a pH within the aforementioned limits. However, the most satisfactory mixture, when buffered to pH 7.6–7.8, caused the isolated chromosomes to swell and become excessively sticky. Moreover, the swelling was accompanied by a fading of internal structure. However, at pH 7.0, the chromosomes closely approached, both in appearance and physical properties, those within the nucleus. It should be noted that the consistency was slightly greater than that of the intact chromosomes. The structure and properties of the chromosomes were maintained throughout the experimental period. The medium used consisted of 0.09 M KCl, 0.06 M NaCl, and 0.005 M phosphate buffer, pH 7.0.

Observations and micromanipulation experiments were routinely made with a Leitz microscope using a 1.8 mm. oil immersion objective, N.A. 1.25, and a 10 × ocular. A micrometer ocular was used for measurements.

OBSERVATIONS

The chromosomes in the fresh cell in Ringer's solution or hemolymph

The chromosomes and nucleolus in the fresh cell were always plainly visible, the bands being sharply defined and either beaded or homogeneous in appearance. A hyaline material, variously referred to as nuclear sap, nucleoplasm, or karyolymph, separated the chromosomes from each other. It has been shown (Glancy, 1940) that this hyaline material is differentiated into a central portion of jelly-like consistency in which the chromosomes are embedded, and a more fluid peripheral zone. The evidence for this regional differentiation was based on experiments involving the injecting of oil drops and carbon particles as well as the manipulation of the chromosomes. The hyaline material is referred to in this paper as the nuclear matrix.

Stickiness. It was found possible to insert microneedles into the nucleus and to force the chromosomes against each other as well as against other structures in the nucleus, and also to push them against injected oil drops and carbon particles. Two chromosomes pushed together by means of microneedles could easily be separated again without sticking to each other. It should be noted, however, that a narrow hyaline zone, presumably the jellied nuclear matrix, always remained between them, thus probably preventing actual contact of the two chromosomes. Similar results were obtained when a chromosome was forced against the nucleolus or the nuclear membrane.

A micropipette containing an aqueous carbon suspension was inserted as close as possible to a chromosome without actually touching it, and a small quantity of the suspension injected. In none of the attempts were carbon particles seen to adhere to the chromosomes, nor did they ever make contact with a chromosome. However, injection of a large amount of fluid caused liquefaction of the jelly matrix, thus permitting the carbon particles to make contact with the chromosomes. In a similar manner, various oils; namely, Nujol, olive, almond, and peanut oils, were injected into the nucleus. None of the oil drops adhered to the chromosomes.

When the cytoplasm was torn without tearing the nucleus, a change occurred in the nucleus. This was made evident by the marked shrinkage of the chromosomes. Two chromosomes pushed together in the nucleus of such a torn cell invariably stuck to each other, and thick viscid strands were pulled out when attempts were made to separate them. The chromosomes also adhered to the nucleolus, the nuclear membrane, injected oil drops, carbon particles, and the microneedles. In every case, separation could be achieved only when accompanied by thick viscid strands pulled out from the chromosomes.

In summary, these experiments show that the chromosomes, because they are prevented from coming in contact by the jelly-like nuclear matrix, appear to be non-sticky in the intact cell. Liquefaction of this matrix occurs when the cytoplasm is torn, thus permitting the chromosomes to make contact. They are then found to be sticky.

Consistency. The micrurgical experiments to be described indicate that, compared to the cytoplasm and the nuclear matrix, the chromosomes are relatively soft, easily deformable gels, and that there are differences in consistency between the bands and the interband regions.

A very finely tapered microneedle was pushed up through the cytoplasm and through the nuclear membrane, into the interior of the nucleus. The needle was pushed in from the side, and even before it was brought into actual contact with a chromosome, the surface of the chromosome became indented on the side next to the needle. The needle was pushed still farther and when its tip went into the interior of the chromosome, the indentation disappeared and the original contour of the chromosome was restored. This deformation of the chromosome must be due to the central jelly-like matrix since insertion of the needle into the more fluid peripheral region of the nucleus had no such deforming action on the chromosomes. The indentation of the chromosome, when the needle tip was being brought into its vicinity, and the readiness with which the chromosome returned to its original shape after the needle had penetrated indicated that the chromosome was of a softer consistency than the surrounding central nuclear matrix.

The relatively low consistency of the chromosomes compared to the central nuclear matrix was also shown by experiments in which oil drops were injected into the nucleus. The effect of the oil drops in causing deformation of an adjacent chromosome differed according to the site of injection. Drops of Nujol, olive, almond, and peanut oils, 1–20 μ in diameter, were injected close to a chromosome. Large oil drops carefully injected into the central jelly-like nuclear matrix produced a rounded depression in the nearest chromosome. However, this did not occur when the matrix was agitated by the needle, thus showing the thixotropic nature of the jelly matrix. Peripherally injected oil drops were never observed to cause deformation. These results indicate that the consistency of the central nuclear matrix is greater than that of the chromosomes, whereas the reverse is true in the peripheral region of the nucleus.

The nucleolus was found to be the most easily deformed body in the nucleus. This was shown by forcing the nucleolus against the nuclear membrane, the chromosomes, and the microneedles. In all cases the nucleolus was deformed in the manner of a fluid drop. When the nucleolus was separated by a microneedle from the small fourth chromosome to which it is normally attached in Chironomus, the nucleolus became spherical.

Evidence for differences in consistency between the bands and the interband regions was obtained in several ways. A microneedle inserted into a chromosome and then withdrawn produced a space which closed over more rapidly in an interband region than in a band. The thin bands were intermediate between the interband regions and the thick bands in this respect. The longer persistence of the hole in the bands is indicative of a higher consistency. The greater resistance of the bands, especially the thicker ones, to deformation during manipulation of the chromosomes, as in the stretching experiments, also is in accord with their higher consistency. The results of injecting oil drops into the chromosomes led to similar conclusions. Of the various oils used, Nujol, olive, almond, and peanut oils, only the last two could be readily injected into a chromosome. This was easily done in an interband region, but only with difficulty or not at all in a band. The injected oil drops became spherical, showing the relatively low consistency of the interband regions, and caused the displacement of the relatively viscid bands on either side.

Extensibility and elasticity. Observations on extensibility and elasticity were made by stretching a small portion of a chromosome between two microneedles. The degree of stretching which could be performed, however, was limited since the nuclear membrane was torn when the microneedles were moved more than approximately 20–30 μ apart. In all cases in which a part of a chromosome was stretched within the nucleus, release from the needles resulted in a complete return to the original appearance.

A short region of a chromosome was seized lengthwise between two microneedles and the needles were slowly drawn apart. As the chromosome was stretched, there was a decrease in its diameter, and its structure became less distinct. The thick bands were more resistant to stretching than the thinner ones, whereas the interband regions stretched most readily. During the stretching the broad bands often separated into several narrow bands, thus demonstrating their compound nature. Faint longitudinal striations which followed the lines of tension generally appeared in the interband regions when a chromosome was stretched more than twice its length. These stress lines became more pronounced as stretching was increased. They did not appear to be identical with the delicate longitudinal striations occasionally seen in unstretched fresh chromosomes. On release of tension, the striations disappeared and the chromosome, even after being stretched five times its length, returned to its original condition. It was not found possible to stretch a given region more than this without tearing the nuclear membrane. In every case, the chromosomes recovered completely after the microneedles were removed.

Lateral stretching of the chromosomes was also performed. Two microneedles were inserted into a chromosome close to the margins, then slowly drawn apart.

The chromosome cylinder flattened during the process and bands which had appeared homogeneous became beaded (Fig. 1). As tension was increased, stress lines, radiating from the point of each microneedle to the chromosome, became visible. On release of tension these lines disappeared, and the beaded appearance of the bands was lost. A chromosome could be stretched approximately three times its original width and recover fully.

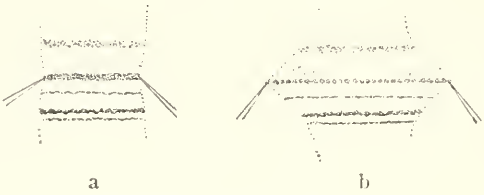


FIGURE 1a. Sketch of a portion of a chromosome showing two microneedles inserted directly into a band prior to lateral stretching of the chromosome within the intact cell. All sketches are of Chironomus chromosomes.

FIGURE 1b. Beaded appearance of the band when the chromosome in (a) is stretched laterally. Note tension lines in the interband regions.

The chromosome membrane. The micrurgical evidence to be presented indicates that the salivary chromosome possesses a delicate elastic membrane. A microneedle was inserted into the margin of a chromosome within the intact nucleus, in an attempt to remove any existing membrane. When the needle was slowly and carefully withdrawn, faint lines were seen radiating from it to the chromosome (Fig. 2a). To test whether or not these lines were actually folds in a membrane which had been lifted off from the chromosome the same operation was repeated and an aqueous carbon suspension was injected into the triangular area in question. The carbon particles converted the region showing the radiating lines into a sac-like structure which remained connected to the chromosome (Fig. 2b). When the micropipette was carefully withdrawn, the fluid evidently escaped from the area, as the sac slowly contracted and the carbon particles came closer to the chromosome where they became packed against its surface (Fig. 2c). Repetition of the

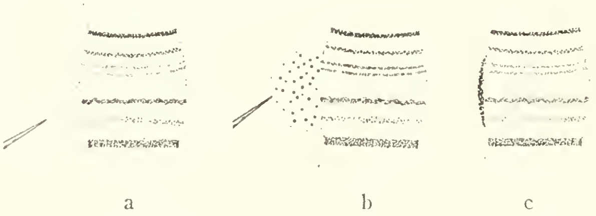


FIGURE 2a. Lifting by a microneedle of a membrane-like material from a portion of a chromosome within the intact cell.

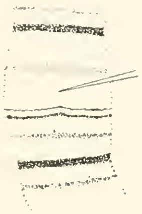
FIGURE 2b. Micro-injection of a carbon suspension into the area subjacent to the lifted membrane shown in (a).

FIGURE 2c. Concentration of the carbon particles into a narrow zone as a result of gradual contraction of the membrane.

experiment injecting a larger quantity of the suspension into the triangular area first converted it into a sac; then as injection was continued, the sac burst, and the carbon particles scattered in the matrix where they briefly exhibited Brownian movement.

Oil drops were also injected in a similar manner into the triangular area produced by pulling on the margin of the chromosome. After the withdrawal of the micropipette, the oil was gradually drawn to the chromosome surface where it remained as a slight protuberance. Larger oil drops were injected and could be seen to be held against the chromosome by what appeared to be a membrane. No deformation of the chromosome occurred, presumably because there was no jelly-like matrix between the oil drop and the chromosome.

Various salt solutions (0.01–1.0 M NaCl, KCl, and CaCl$_2$) and also distilled water were injected into an interband region in an amount which caused a swelling of the region without breaking its contour. Figures 3a, b, and c show the condition of the chromosome before, during, and after the injection. With the progress of the injection, the lesser bands in the vicinity faded out and the zone underwent swelling until a heavy band on each side of the injected zone was reached. The injection was continued within the limit of bursting. The pipette was then removed, and the swollen zone returned to its original dimensions in which the final appearance was that of the region prior to injection. The surface of the injected region could be ruptured when the amount of fluid injected was sufficient to cause it to swell to approximately two and a half times its original diameter. Thereupon the membrane-like boundary suddenly disappeared irrespective of the fluid injected. When CaCl$_2$ was used a granulation appeared in the nuclear matrix around the injected zone (Fig. 3c). This did not occur with water, NaCl, or KCl solutions.



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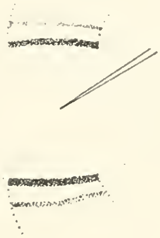

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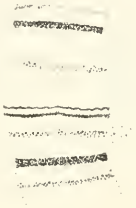

c

FIGURE 3a. Micropipette inserted into an interband region of a portion of a chromosome within the intact cell showing the initial stage of injection.

FIGURE 3b. Localized swelling and loss of structure in the injected portion of the chromosome. Results are similar with solutions of NaCl, KCl, CaCl$_2$, and distilled water.

FIGURE 3c. The appearance of the same chromosome after injection of sufficient CaCl$_2$ solution to rupture the injected region. The bands have reappeared in their original positions, but the distinct boundary (membrane) shown in (a) is no longer evident. Note the granular region of the nuclear matrix.

It may be concluded, therefore, that the salivary chromosome possesses a delicate elastic membrane.

Internal Structure. The visible structure of the chromosomes was studied by careful and extended observations of the chromosomes within the intact nucleus both of the isolated gland and of glands within the intact larvae.[2] For this purpose the observations were made with a Leitz 1.4 N.A. apochromatic oil objective and a triple lens condenser. The equipment was sufficient to resolve the hexagonal apertures of the *Pleurosigma angulatum*. The distinct and sharply defined bands were beaded in some regions, and homogeneous in appearance in others. The interband regions, which were much less refractile, generally appeared either homogeneous or finely granular. In unusually large chromosomes the granulation was coarse and often the arrangement gave the effect of longitudinal striations. These granular striations, which were seen at all levels in the chromosomes, were individually connected to the beads in the bands. During these observations the chromosomes were not under tension and in the case of the isolated gland were free from compression.

That the salivary gland chromosome is made up of numerous longitudinal fibrils was further indicated by microdissection. The chromosome membrane was first removed by a very fine microneedle over a length of several bands from the region to be studied. The needle was then inserted into the margin of an exposed interband region. This adhered to the needle and, when the needle was carefully moved to one side, a strip of very thin fibril with one end on the tip of the needle could be seen being carried away from the chromosome. As the fibril was being carried still farther it was separated from the chromosome over a length of several bands (Fig. 4).

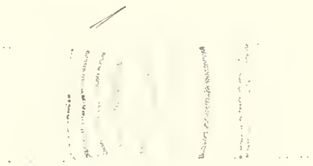

FIGURE 4. A single fibril with a chromonema-like appearance being removed by a microneedle from a chromosome within the intact cell.

The isolated fibril showed nodular swellings which corresponded in position with the bands from which it was being separated. This was done over and over again with the same chromosome and with different chromosomes. The same phenomenon could be observed whether the bands on the operated chromosome were homogeneous or beaded. The fibrils could be stretched and, when tension was released, they returned to their original lengths. One case was observed in which a fraying of the chromosome resulted when a pipette had been inserted to inject water. The injection was made close to the periphery and the pipette then removed. These partially isolated fibrils closely resembled that in Figure 4. The microdissection experiments were performed more readily with large chromosomes in which longitudinal striations were visible. They were also done with the chromosomes in which the interband regions appeared homogeneous. The dissection evidence strongly suggests that longitudinal fibrils possessing nodular swellings really exist in the chromosomes.

The state of the chromosomes in nuclei rendered optically homogeneous by alkaline or hypotonic Ringer's solution. The question has frequently been raised whether chromosomes in so-called hyaline nuclei maintain their morphological integrity or whether they lose their identity through dispersion in the hyaline phase. Naturally occurring hyaline salivary gland nuclei were never found, but could be readily produced by immersion of the gland in slightly hypotonic or in mildly alkaline (pH 8.4) Ringer's solution. Within a few minutes after immersion the nucleus swelled, chromosomes faded, the interbands disappearing first, and finally only the nucleolus remained visible. When the gland was returned after several minutes to isotonic Ringer's solution at pH 7.0, the chromosomes reappeared.

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[2] As Buck (1938) has already described, the intact larvae can be mounted between slide and coverslip and because of the transparency of its body wall, the cells in the salivary glands are easily visible under high power, and can be satisfactorily observed. Their appearance was identical with that of the isolated glands mounted in Ringer's solution.

A microneedle could be inserted into one of these hyaline nuclei without causing its collapse and usually without producing any visible effect. In some cases, however, the nuclear membrane wrinkled slightly when the microneedle was withdrawn, indicating that fluid was lost. When a microneedle was moved slowly back and forth within the nucleus no regional resistance to its movement was encountered. Similarly no resistance was offered to movement of the nucleolus throughout the entire nucleus. Return of the gland to Ringer's solution caused the chromosomes to reappear. It is noteworthy that the chromosomes which reappeared were frequently distorted in shape.

Carbon particles injected into the center of an untreated nucleus remained at the site of injection presumably because they were caught in the central jelly matrix in which the chromosomes are embedded. However, an injection made just under the nuclear membrane of an untreated nucleus, where the nuclear matrix is relatively fluid, resulted in a scattering of the carbon particles between and around the parts of the chromosomes in the peripheral region. None of the particles penetrated the central region of the nucleus. Exposure of such an injected nucleus to an alkaline or hypotonic Ringer's solution or to distilled water, caused the chromosomes to disappear. The fact that the chromosomes still retained their identity and did not go into solution was shown by the alignment of the carbon particles which kept their relative positions outlining the contiguous borders of what must be the swollen chromosomes (Fig. 5). The carbon particles failed to become completely dispersed as would be expected if the chromosomes had gone into solution. In nuclei which were first made hyaline, then injected with the carbon suspension in the peripheral region, a similar alignment of the carbon particles resulted. In both cases the chromosomes reappeared in the expected positions when the glands were placed in Ringer's solution.

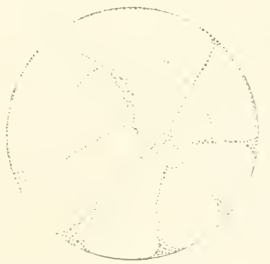

FIGURE 5. Alignment of carbon particles outlining the contiguous borders of adjacent chromosomes in an optically homogeneous nucleus. The nucleus was first injected with a carbon suspension, then hyalinized by immersion of the gland in hypotonic Ringer's solution.

It may be concluded, therefore, that although moving microneedles back and forth within the hyaline nucleus fails to reveal the presence of the swollen chromosomes, nevertheless the chromosomes have maintained their morphological integrity as shown by the injection of carbon particles.

Isolated chromosomes

The physical properties of the isolated chromosomes may be seriously affected by the methods used in their isolation and by the medium in which they are maintained. In the following experiments, attempts were made to maintain the properties which had previously been found for the chromosomes within the nucleus. It was found that rapid isolation of the chromosomes without contact with the torn cytoplasm was essential to prevent changes in structure and physical properties. Chromosomes which remained in the torn cell or came in contact with the cytoplasm during the isolation were shrunken, often distorted, and highly sticky. Of the various media tried, the one which caused the least change in the chromosomes after their isolation was the neutral KCl–NaCl solution described in the section on methods. In the experiments which follow on the isolated chromosomes, it is understood, unless otherwise stated, that the chromosomes were isolated into the neutral KCl–NaCl solution without coming in contact with the torn cytoplasm, transferred to a fresh drop of the same solution, and then manipulated. The duration of an experiment was not more than 10 minutes.

Stickiness. Chromosomes isolated into the neutral KCl-NaCl solution. Ringer's solution or hemolymph, were not unduly sticky. It was found that alkalinizing the medium (pH 8.0) caused the chromosomes to become sticky, whereas acidifying the medium (pH 6.5) reduced stickiness.

Consistency. The consistency of the isolated chromosomes in slightly alkaline KCl–NaCl solutions, pH 7.6–7.8, was found to resemble most closely that of the chromosomes in the intact cell, but structure was indistinct and the chromosomes were sticky. As the pH was decreased, the chromosomes shrank and the consistency increased. In neutral solutions little shrinkage occurred and the consistency was but slightly greater than that of the chromosomes within the intact cell. In acidic solutions (pH 6.0–6.5), the effects were more marked. At corresponding pH values, the consistency was always somewhat greater in Ringer's solution and in hemolymph than in the KCl–NaCl medium.

Chromosomes maintained in the neutral KCl–NaCl solutions were, like those in the intact nucleus, soft easily deformable gels which could be compressed without buckling. When they were cut or pulled apart by microneedles the cut or broken ends assumed smooth, rounded contours. This is in marked contrast to the results obtained with chromosomes which have been isolated after exposure of the glands to osmic acid vapors, formaldehyde, or acetic acid, following the methods of Buck (1942). Even brief treatment has a pronounced effect on the consistency of the chromosomes, causing them to become relatively rigid bodies which break squarely across when stretched.

It may be concluded that, although the consistency of isolated chromosomes maintained in a slightly alkaline KCl–NaCl solution most closely resembles that of the chromosomes within the cell, a neutral medium is preferable for manipulation since structure is still maintained and stickiness is lessened.

Extensibility and elasticity. The use of isolated chromosomes permits unrestricted stretching which obviously is impossible in the intact cell. It was found possible to stretch an isolated chromosome 10 times its original length and obtain complete recovery on release of tension. During the process of stretching, the interbands stretched somewhat more readily and so underwent a greater decrease in diameter than the bands. The wide bands separated into two or more narrow bands as occurred in the intact cell operations. As stretching was increased the diameter of the chromosome decreased so that at 10 fold elongation it appeared to be a mere thread with occasional nodular swellings. If this degree of stretching was not repeated more than 2 or 3 times, it was completely reversible. Repeated stretching caused permanent deformation, particularly of the interband regions, which remained in a partially stretched condition even after tension was released. Much greater stretching was possible without causing a chromosome to break in two. In some cases, a chromosome could not be broken within the limits of stretching of the micromanipulator. Thus one chromosome was stretched 25 fold without breaking. When tension was released, it returned to 1.3 × its original length. Other chromosomes broke in two when stretched 12 fold. The break always occurred in an interband region, although this was not apparent until the chromosome had partially recovered. Fibrillae were occasionally seen at the broken ends. It should be pointed out that greatly stretched chromosomes developed a coarse fibrillar structure and rapidly deteriorated. This fibrillar appearance was different from that observed in unstretched chromosomes in that these fibrils were relatively coarse, and always followed the lines of tension. Deterioration also occurred, although much more slowly, in chromosomes which were not manipulated, indicating that the medium is not all that is to be desired.

It was found that on the addition of acids (pH 6.0–6.5) or calcium ions to the KCl–NaCl medium, the consistency of the bands was increased more than that of the interband regions. This effect was especially marked in the acid solutions, the bands becoming curved or bent rather than stretching or separating into narrow bands when placed under tension. The interband regions, although having a higher consistency than in neutral solutions, nevertheless were readily extensible, and when stretched more than twice their length appeared coarsely fibrous. At a 5–10 fold elongation, a chromosome broke in an interband region as before with fibrillae being evident at the broken ends. In slightly alkaline solutions, pH 8.0, the chromosomes became extremely ductile and showed almost no elasticity. The loss of elasticity was partially prevented by the addition of calcium salts to the alkaline solutions.

Isolated chromosomes were also stretched laterally. These gave results qualitatively similar to those observed in the intact cell. The isolated chromosomes, however, could not be stretched more than approximately double their width without deteriorating. In some instances, at least, the deterioration was associated with tearing the chromosome membrane.

In some of the experiments Buck's (1942) method was used of exposing glands to osmic acid vapors for 18–24 hours at 5° C., then isolating the chromosomes in distilled water. The chromosomes could be isolated readily by this method, and, in agreement with Buck, were tough, relatively rigid bodies, breaking squarely across in an interband region when stretched more than four times their length. Occasional fibrillae appeared extending from the broken ends of the chromosomes. This was also observed by Buck, although he attached no particular significance to it. Stretching was reversible only up to 2 fold elongation (or 100 per cent as stated by Buck). Thus these chromosomes were far less extensible and elastic than those in the fresh state.

Structure. The chromosomes isolated in the KCl–NaCl solution at pH 7.0 generally showed no structure either in the interband regions or in the bands. Occasionally fibrillae were visible in the interband regions. On the other hand, in an acid solution (pH 6.5), fibrillar structure was more generally evident. In those cases where the fibrillar structure was already apparent, it became more distinct when the medium was slightly acidified.

Isolated chromosomes were dissected in the same manner as the chromosomes in the intact cell. The fibrils removed from the isolated chromosomes, behaved, except for a slightly increased stiffness, similarly to those removed from the chromosomes within the nucleus.

Occasionally it was found possible to produce a breakdown of the chromosomes into numerous longitudinal fibrils. This was done as follows: an intact gland was immersed in a hanging drop of neutral KCl–NaCl or in amphibian Ringer's solution, then a large pipette, about 15 μ in diameter, was inserted into the nucleus of an intact cell and a single chromosome withdrawn. This could be done readily, indicating the ease with which the nuclear matrix becomes solated, and the relative non-adhesiveness of the chromosomes to each other. The chromosome was then expelled into the drop to one side of the gland, whereupon it sometimes became shredded into numerous longitudinal fibrils which resembled the fibrils obtained by dissection of an intact chromosome. This operation was successful only if the bore of the pipette was close to the diameter of the chromosome, thus suggesting the possibility that the chromosome membrane may have been removed while the chromosome was being either sucked into or expelled from the pipette. If the pipette was much larger than the chromosome, then the chromosome was expelled intact. The fibrils of the shredded chromosome were very numerous, sometimes appearing as a jumbled mass, and sometimes as a bundle of essentially parallel longitudinal fibrils. This experiment was repeated several times on the same day and on different days. The operation was not successful every time but the fault could be ascribed to the critical bore of the pipette and the speed with which ejection was made. The fraying of these chromosomes occurred irrespective of whether the medium into which they were expelled was NaCl–KCl or amphibian Ringer's solution. The operation was not done a sufficient number of times to discover an optimum medium for the fraying. The fact that it can be made to occur even very occasionally is highly significant.

Discussion

The results of these micrurgical experiments indicate that the normal structure and physical properties of salivary gland chromosomes can be maintained only if due precaution is taken to avoid the deleterious effects of torn cytoplasm. The "acid of injury" reaction of torn cytoplasm, originally described by Chambers (1924b), is significant in this regard. More recently, Duryee (1941) has reported the injurious effects of torn cytoplasm and calcium ions on the isolated chromosomes and germinal vesicles of amphibian oocytes. The changes in structure and physical properties of the salivary gland chromosomes which occur in a torn cell appear to be similar to those effected by acid or calcium ions. This suggests that calcium and hydrogen ions may be released from the injured cytoplasm.

The general finding that chromosomes isolated in media of a physiological pH are sticky raises the question as to why they do not adhere to each other within the normal nucleus. It has previously been shown (Glancy, 1940) that the chromosomes within the salivary gland nucleus are normally held apart by a thixotropic jelly matrix which becomes solated when the cell is torn. Although the chromosomes in the torn cell tend to become progressively sticky, this change is minimized if they are immediately removed from the cell so that contact with the torn cytoplasm is avoided. With prolonged contact, however, the chromosomes become so sticky that it is virtually impossible to manipulate them. The latter observations may indicate that the stickiness of isolated chromosomes is caused in part by chemical substances liberated from the disintegrating cytoplasm.

The reports of various investigators (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; and Pfeiffer, 1940) to the effect that isolated chromosomes are tough, viscid gels, are in contrast to the observations in this paper which indicate them to be soft, easily deformable gels. The discrepancy may be explained in part by the failure of these investigators to observe precautions with respect to injured cytoplasm and the medium. My observations show that the consistency of isolated chromosomes tends to be greater than that of those within the nucleus, especially in the presence of injured cytoplasm. This increase in consistency seems to be associated with a simultaneous shrinkage of the chromosomes. Since the above investigators manipulated the chromosomes in the same drop of hemolymph or Ringer's solution in which the isolation had been performed, it is likely that the drop may have been acid because of the presence of the injured tissues. Even slight acidification of the medium has been shown to cause an increase in consistency. A similar effect results from the presence of calcium ions. Osmic acid vapors, used by Buck (1942) in treating the salivary glands prior to isolation of the chromosomes, cause far greater changes in physical properties although no shrinkage occurs. The findings of Buck, which we have confirmed using his technique, to the effect that the chromosomes are tough, cannot be compressed without buckling, and that they break squarely across when stretched, are at variance with those obtained on fresh chromosomes as shown in this investigation. It is thus evident that the consistency of the chromosomes is influenced by many factors.

The relative consistency of the bands and the interband regions has been discussed ever since Balbiani (1881) first suggested the bands to be gel and the interband regions, sol. Guareschi (1939) has observed that in dark field only the thicker bands are visible, whereas the thin bands and interband regions are not visible, from which he inferred that the former are in a gel state, and the latter in a weak gel, or more probably a sol state. Our microdissection experiments confirm Guareschi's inferences as to these differences in consistency. The thin bands and the interband regions must be weak gels rather than sols, however, for a puncture made in these regions leaves a temporary opening, a condition which does not obtain in a sol. This observation is further strengthened by the high elasticity of the chromosomes for, while gels are generally very elastic, a sol has negligible elasticity.

The extensibility and elasticity of the chromosomes have been studied by numerous investigators. Since it is well known that the properties of a colloidal structure depend to a large extent on the medium, it is to be expected that the conditions under which the chromosomes are examined will influence their degree of elasticity. It is to be noted that the stretching experiments of Vonwiller and Audova (1933), Barigozzi (1938), Stefanelli (1939) and Pfeiffer (1940) were performed in 0.6 per cent NaCl, Ringer's solution, or hemolymph, without regard for the effects of the torn cytoplasm. These investigators found that the chromosomes could be stretched many times their length, though not reversibly. Pfeiffer and Stefanelli found stretching was not reversible beyond a 2 fold elongation as did Buck (1942) using osmicated chromosomes. It has been shown in this investigation that the elastic limit of fresh chromosomes in the intact cell is at least 5 fold, and when isolated in a KCl/NaCl medium, as much as 10 fold. This unusually high elasticity indicates the presence of long fibrous molecules (Mark, 1941) of which nucleoproteins are a characteristic type (Mirsky and Pollister, 1942).

The presence of a membrane on the salivary chromosomes has been described heretofore from the appearance of fixed and stained chromosomes (Schultz, 1941; Kodani, 1942). Metz (1934) believed the presence of a sheath or membrane to be necessary on theoretical grounds. Painter (1941) originally considered a pellicle to be present, but finally concluded, after studying the action of alkaline solutions on the salivary chromosomes, that the chromonemata are held together by a matrix, and that a pellicle is lacking. That the fresh chromosome possesses a membrane is strongly suggested by my microdissection experiments and observations that the outer boundary of the chromosome ruptures at a critical diameter when stretched by fluid injections. The membrane may aid in preventing contact between the chromosomes, since they tend to become sticky if it is torn, or it may serve to hold the chromonemata together.

The question as to whether or not the chromosomes in hyaline nuclei maintain their morphological integrity has frequently been raised. Stefanelli (1939) and Guareschi (1939), studying salivary gland nuclei rendered hyaline by immersion of the gland in hypotonic solutions, concluded that the chromosomes are completely dispersed. Stefanelli's conclusions, based primarily on the observations that microneedles can be moved back and forth within the hyaline nucleus without meeting resistance and that the nucleolus can be displaced without rebounding, do not conclusively demonstrate that the chromosomes are dispersed, however. The failure to find evidence for chromosomes by this technique might be explained in several ways: first, movement of the microneedles within the thixotropic nucleus might well induce solation; secondly, the consistency of the nuclear matrix in such nuclei may be close to that of the chromosomes; and thirdly, the swollen chromosomes are closely pressed against the nuclear membrane, leaving the center of the nucleus almost devoid of any resisting structure. To Guareschi's conclusions, it may be objected that a dilute gel cannot necessarily be distinguished from a sol under dark field illumination, and secondly, that the refractive indices of the colloidal particles in the chromosomes and the surrounding nuclear matrix may have been changed by the hydration which occurs in hypotonic solutions. Micro-injection results on salivary gland nuclei, likewise made hyaline by hypotonic solutions, indicate that the chromosomes, though invisible, maintain their morphological integrity, since injected carbon particles clearly indicated the outlines of the chromosome cylinders. If the chromosomes were completely dispersed, the injected carbon particles should be distributed at random in the nucleus.

The polytene concept of chromosome structure rather than the alveolar is supported by the various observations made in this investigation. The controversy regarding the structure of the salivary chromosomes results in part from the fact that fixed and stained chromosomes do not always present a similar appearance with regard to the finer details. Since the striations are especially evident in stretched chromosomes, Metz (1941) believes them to be artifacts produced by drawing out the walls of alveoli. Buck (1942) has provided experimental support for the alveolar hypothesis. It is agreed that a fibrillar-like appearance in homogeneous-appearing chromosomes can be produced by stretching. However, I have never observed a pre-existing alveolar structure in fresh chromosomes, nor does Buck state that the alveolar condition was present before he treated the glands with osmic acid vapors, although he does state that the treated chromosomes closely resemble those in the living animal. Most of his photomicrographs fail to show an alveolar structure. It is conceivable that stretching the chromosomes could cause aggregation of delicate, scarcely visible or non-visible fibrils, into the relatively coarse fibrils which are commonly seen in stretched chromosomes. Buck tried unsuccessfully to shred the chromosomes into fibrillae. This may easily be accounted for by the fact that he used osmicated chromosomes which are of such high consistency that shredding is impossible.

A fibrillar appearance was occasionally noted in the fresh salivary chromosomes studied in this investigation. Buck (1942) also reported that fresh chromosomes may occasionally show longitudinal striations, but he attached no particular significance to this observation. In chromosomes which show no structural details in the interband regions, a slight shrinkage produced by mild acidification (pH 6.5) results in the appearance of delicate longitudinal striations but never alveoli. Since the chromosomes have not been stretched, it does not seem likely that the fibrillar appearance can be explained on a basis of drawn out alveoli. The observation that fibrillae may appear at the broken ends of greatly stretched chromosomes may be interpreted in two ways, as Buck (1942) pointed out when he made a similar observation on osmicated chromosomes. The fibrillae may have been pre-existing, or they may be artifacts produced by the stretching. More significant is the microdissection from fresh unstretched chromosomes, either within the intact nucleus or after isolation, of delicate longitudinal fibrils which give the appearance of chromonemata. It is difficult to see how such fibrils could be removed from an alveolar mesh. Attempts to remove transverse fibrils, which should be equally possible if an alveolar structure exists, were uniformly unsuccessful. The finding that a fresh chromosome, expelled from a micropipette into Ringer's or KCl–NaCl solution, may break up into numerous delicate longitudinal fibrils, but never an alveolar mesh, is also difficult to explain except on a polytene basis.

Summary and Conclusions

Micrurgical experiments performed on the fresh salivary gland chromosomes of mature Chironomus larvae within the nucleus of the intact cells and after their isolation into a specially designed medium indicate the following:

1. Puncture of the cell with a fine microneedle has little or no effect on the structure and physical properties of the chromosomes provided tearing of the cytoplasm is prevented.

2. Tearing of the cytoplasm causes the chromosomes to shrink markedly and become highly viscid.

3. The structure and physical properties of chromosomes isolated into a medium consisting of 0.09 M KCl, 0.06 M NaCl, and 0.005 M phosphate buffer at pH 7.0, are not appreciably altered, except for a slight increase in consistency, from those of chromosomes in the intact cell if precaution is taken to prevent contact of the chromosomes with the torn cytoplasm.

Physical properties

4. Evidence for stickiness of the chromosomes can be obtained only when the thixotropic jelly matrix surrounding them is solated.

5. The chromosomes are soft, easily deformable gels with interband regions of lower consistency than the bands.

6. The chromosomes are highly extensible and elastic. They can be stretched 5 fold within the intact cell without permanent deformation. Greater stretching cannot be accomplished without tearing the nuclear membrane. Isolated chromosomes regain their initial length after 10 fold elongation, but excessive stretching (12–25 ×) causes the chromosomes to break in an interband region.

7. Modifications in the properties of the chromosomes are easily induced by chemical agents. Dilute alkalies decrease consistency and elasticity, but increase extensibility. Dilute acids and calcium ions increase consistency, and decrease extensibility and elasticity. Osmic acid and formalin have a similar but much greater effect.

8. Chromosomes in nuclei hyalinized by hypotonic or alkaline Ringer's solution retain their morphological integrity.

Structure

9. The fresh chromosome possesses a delicate elastic membrane.

10. Chromosomes, either in the intact nucleus of the unoperated cell, or when isolated, occasionally display delicate longitudinal striations in the interband regions, but never show alveoli.

11. Slight acidification of the medium to pH 6.5 causes the appearance of similar striations in the interband regions of unstretched chromosomes.

12. Chromosomes broken in two by stretching frequently exhibit fibrillae at the broken ends.

13. Delicate fibrils may be dissected from the intact or isolated chromosome. The fibrils possess nodular swellings at intervals corresponding to the bands.

14. A chromosome drawn up into a micropipette of optimal size, then expelled into Ringer's or KCl–NaCl solution, may shred into numerous fibrillae.

These observations are best interpreted as supporting a polytene concept of chromosome structure.

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Titles recorded alphabetically with initial dates.

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[ ] incomplete.

+ receive currently.

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THE SWIMMING OF LEPTOSYNAPTA

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The fact that young adult leptosynaptids swim, or pass through a post-larval swimming stage, has not been adequately recorded, and is not generally recognized by the students of the Holothuria. Adult leptosynaptids are usually considered to be exclusively bottom-dwelling forms, and the only reference to the contrary that I have found is a brief and incomplete statement by Clark (1907) to the effect that: "Few, if any, synaptids swim, except in the larval state, but young ones, up to 2 cm. in length, are sometimes found floating, *if not swimming,* in the water." The italics are mine. I am therefore placing on record the following observations on the swimming habits of these animals, made during the summers of 1944 and 1945 at Woods Hole, Mass. I observed the same phenomenon a few years earlier (in 1939), but no detailed records were made at that time.

On August 14th, 1944, between 9:15 and 9:30 P.M., while collecting the swarming heteronereis form of *Nereis limbata* in Eel Pond, ten small, colorless, transparent holothurians were observed swimming more or less aimlessly near the surface of the water. They were being carried partly by their own movements and partly by the tidal currents, across the field of light cast on the water by the 100-watt bulb of the Nereis collecting lamp. All ten were collected. These swimming animals were 30 to 50 mm. in length and about 3 mm. in diameter. When touched in the water by the cheesecloth surface of the Nereis collecting net, they ceased their swimming movements and contracted, shrinking to about one-fourth of their extended length by the expulsion of fluid, became opaque, and sank slowly toward the bottom. If collected by being dipped up with a quantity of sea water in a finger bowl without being disturbed, they continued their swimming movements in the dish. Jarring of the dish caused extrusion of fluid, contraction, and sinking of the animal. The change from the glass-like transparency of the extended form to the white opacity of the contracted form is extremely striking.

The animal swims only when in the extended state. The effective stroke occurs after the animal has assumed the shape of a wide U (see Fig. 1). The longitudinal muscles on one side of the body suddenly contract, drawing the posterior end of the body up to and past the tentacled anterior end, and then, as suddenly, relax. Relaxation results in the resumption of the U-shaped form, and is followed by another contraction of the longitudinal muscles. A series of these contractions creates an effective swimming movement, comparable to the breast-stroke of human swimmers, in the direction of the base of the U. If, on one stroke, the head crosses above the posterior region, on the next it usually crosses below, so that there is an alternation of movements, presumably brought about by the contraction of sets of longitudinal muscles of opposite sides of the body. At the same time, the whole body turns frequently, so that locomotion is not in one direction, but is more or less aimless. Nevertheless, the swimming leptosynaptids can move across a ten-inch finger bowl in the course of four or five minutes.

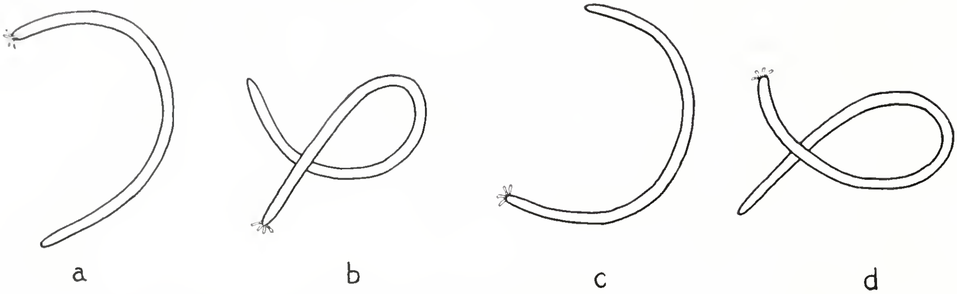

FIGURE 1, a, b, c, d. Successive stages of swimming movements of *Leptosynapta inhaerens*. The effective stroke, directed toward the base of the U, occurs between a and b, and between c and d. During the period between b and c, there has occurred a rotation of the entire body, on the axis of the U, through 180 degrees.

On the following evening (August 15) nine more leptosynaptids were observed swimming in Eel Pond between 9:25 and 10:00 P.M., and were collected. At 10:00 P.M., on entering the darkened laboratory, I observed that four of the leptosynaptids collected the previous evening (and left in a bowl of standing sea water on the sea water table since that time) were in the extended condition and undergoing rapid swimming movements. These animals had remained in the contracted state at the bottom of the dish during the day (see Fig. 2). Similar observations were made for these and other animals on subsequent evenings. Swimming is undoubtedly a

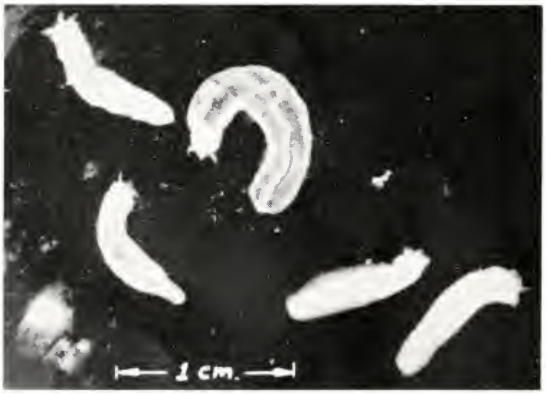

FIGURE 2. Photograph of five *Leptosynapta inhaerens*, living young adult "swimming forms." Four are in the completely contracted, opaque state; the fifth almost completely contracted, and partially transparent. Centimeter scale indicated below.

dark-conditioned phenomenon, yet placing leptosynaptids in the darkroom during the day did not induce swimming. Presumably either a certain period of illumination or of complete rest is first necessary. Quatrefages (1842) early called attention to the greater general activity of Leptosynapta at night as compared with that during the day. On the morning of August 16th, at 11:00 A.M., one leptosynaptid in a large finger bowl (covered with cheesecloth, over which a slow stream of sea water was flowing) was still swimming, but did not show the alternate criss-crossing of the two ends as observed in nature. This is the only Leptosynapta observed swimming during the day.

A complete record of Eel Pond collections is as follows:

Date	Time	Number collected
Aug. 14, 1944	9:15– 9:30 P.M.	10
15	9:25–10:00	9
16		2
17	9:10–9:40	5
18		none
22	10:00 P.M.	1
23	9:30 P.M.	2
28		none
29		"
30		"
31		"
Sept. 4		"
6		"
7		"
	1944 Total	29
Aug. 25, 1945	10:30 P.M.	1
Sept. 6	9:00 P.M.	2
7		1
8		1
9	8:45 P.M.	1
	1945 Total	6

The 1945 specimens were collected by Miss Catherine Henley and Miss Roberta Lovelace. Nereis collections were made almost every night from early June until the end of September, 1945, but no leptosynaptids were seen other than the six listed above. The last specimens taken in 1944 were larger than those first collected. Those kept for two or three weeks in the laboratory grew slightly and showed an increase in number of tentacular sensory cups. These facts indicate the probability that Leptosynapta swims only during a limited period of its young adulthood.

An examination of the collected specimens revealed only a few grains of sand, and some diatoms and other debris in the intestine. Presumably a necessary condition for swimming is absence of intestinal sand which would prevent floating. When the animals swell to the extended condition, the intestine is not filled with water. The intestine can be seen within the body and is approximately of normal diameter. The water probably enters the perivisceral cavity. Tests were made to ascertain the places of exit of the fluid from the body by dropping extended animals into a suspension of Chinese ink in sea water. These tests were somewhat inconclusive, but indicated a general release of fluid along the major portion of the body surface, rather than any markedly localized release.

A preliminary examination of the anchors, anchor plates, and other spicules indicated size characteristics somewhat intermediate between those of the two common Woods Hole species, *Leptosynapta inhaerens* and *Leptosynapta roseola*. The radial plates of the calcareous ring were seen to be perforated. Some of the material was then sent to Dr. Elisabeth Deichmann, who kindly identified the swimming forms as *Leptosynapta inhaerens* (O. F. Müller). This species, as found in the Woods Hole region, was described by Clark (1899). The deviations from the typical adult characteristics which were found in the swimming forms were deviations characteristic of young individuals.

Leptosynaptas have apparently never before been seen swimming in Eel Pond, even by such a veteran collector as Mr. George M. Gray. Fish (1925), who studied the extensive plankton collections of the Woods Hole Fisheries Laboratories, records the presence of only one leptosynaptid—as follows: "A specimen of *Leptosynapta inhaerens* (Müller), 20 mm. long, was taken on Sept. 19 after a hard northeast storm. This was not a free-swimming form and would not normally occur in surface collections." Dr. George L. Clarke, of the Woods Hole Oceanographic Institution (personal communication), has never taken a holothurian in his plankton hauls.

Swimming of certain other holothurians is not unknown. There is a pelagic form, *Pelagothuria natatrix*, from the Eastern Pacific, described by Ludwig (1893). For *Stichopus natans*, swimming movements were observed by Sars (quoted by Ludwig, 1889–92). Prior to the present work these constituted the rare exceptions to the general rule that adult holothurians do not swim.

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CORRELATION BETWEEN THE POSSESSION OF A CHITINOUS CUTICLE AND SENSITIVITY TO DDT *

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INTRODUCTION

The remarkable insecticidal qualities of 2, 2-bis (p-chlorophenyl) 1, 1, 1, trichloroethane, more commonly called "dichloro-diphenyl-trichloroethane" or just "DDT," were discovered by Paul Müller of the J. R. Geigy Company of Switzerland a little more than five years ago (Lauger, Martin, and Müller, 1944). The first samples reached this country in August, 1942. Researches in Switzerland, Germany, England, and the United States, accelerated by war-time urgency, have developed a voluminous amount of data. Most of these data are purely practical and will not be reviewed here; [2] further, these economic reports and papers are appearing so rapidly that it is very difficult to separate fact from fancy in the confusing maze of seeming contradictions. Certainly numerous popular articles and stories are dangerously misleading (see, e.g., warning by Cox, 1945, and Anonymous, 1945). The fact remains, however, that DDT is a remarkably potent insecticide and that from the human point of view it can be used with relative safety for certain insect pests (flies, mosquitoes, lice, bedbugs, etc.).

Since this new compound is so extremely toxic to many insects, one is naturally led to inquire what its effects are on other animals. Both a desire to find more favorable animals for studying the physiological effects of DDT, and a need to evaluate the effects of the compound on the entire biota of natural waters, led to determinations of the toxicity of DDT to representatives of various animal phyla. During the course of making these determinations it was noted that animals with exoskeletons, especially chitinous exoskeletons, were notably susceptible. Experiments were accordingly performed to test the validity of this correlation. From these experiments it was concluded that the correlation is valid. A hypothesis is developed in this paper that chitinous cuticles selectively concentrate DDT from the bathing media by adsorption and result in a higher concentration of the toxin inside the animal. There is good evidence to indicate that the relation of the chitinous cuticle to the selective action of DDT is no more than that of a concentrating mechanism. The actual lethal action of DDT seems to be another problem (see, e.g., Yeager and Munson, 1945).

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[2] Large numbers of papers can be found, especially in the February, 1944 and June, 1945 issues of the *Journal of Economic Entomology*. The review in *Soap* is a useful digest (Anonymous, 1945).

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania, and, later, the University of Minnesota. Most of the data were incorporated in a paper presented at the meetings of the Entomological Society of America in New York City, December 14, 1944.

Materials and Methods

For the purpose of this study only aquatic species or aquatic stages were employed. The toxin was applied as what seems to be a collodial suspension in the bathing medium because the solubility of DDT in water is very low, at room temperatures too low to show significant toxicity to susceptible animals. Mechanically shaking DDT in distilled water (68 hours), filtering, and then centrifuging with an angle centrifuge to remove minute particles (15,000 G for one hour),[3] gave a solution that slowly killed about 50 per cent of the mosquito larvae tested at 15° C. but had no significant effect on larvae at 28° C. Several slightly different tests gave similar results. Comparing these data with data from bio-assay of known dilutions of collodial suspensions prepared from acetone solutions, we concluded that the solubility of DDT in water probably lies between 0.2 and 1.0 parts per billion when prepared by agitation in water.[4] Presumably suspensions from acetone solutions would give much higher solubility values, perhaps one hundred times as high (Lewis and Randall, 1923). The exact physical state, then, of the preparations we are calling "suspensions" is uncertain but at least the preparations are sufficiently homogeneous to dilute satisfactorily.

The suspensions were prepared by adding a known volume of an acetone solution of DDT beneath the surface of a known volume of water (distilled, fresh, or sea water). A suspension of one part per million that will give reproducible results and dilute satisfactorily can be prepared by adding one ml. of a 0.1 per cent DDT solution beneath the surface of a liter of water. Stronger suspensions were usually made from more concentrated DDT solutions. Suspensions as strong as one part per hundred thousand are not stable and do not dilute satisfactorily.

Suspensions of one part per million in sea water diluted with distilled water and tested by bio-assay with mosquito larvae indicated that the suspensions in sea water have essentially the same characteristics as those in fresh and distilled water.

Since all suspensions contained a small amount of acetone, at least two controls were always used. One was a control in the water indicated; the other, the same plus whatever was the highest per cent of acetone in any suspension. In general, most animals were indifferent to 0.1 per cent acetone. The few cases where 0.1 per cent acetone seemed to have a possibly deleterious effect (e.g., certain Coelenterata), tests were repeated with lower acetone concentrations. The more dilute suspensions, prepared by dilution of a one part per million suspension containing 0.1 per cent acetone, had only negligible amounts of acetone.

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[3] This is a force sufficient to sediment bacteriophage particles of approximately 20 $m\mu$ diameter but should not affect molecules several times larger than the molecule of DDT.

[4] This is very much lower than the one part per million figure given by Neal *et al.* (1944). Our bio-assay data, as well as similar data from other laboratories, are not consistent with this high estimate.

The amount of fluid used varied with the size of the animals. Protozoa and certain other forms were tested in watch glasses containing 10 ml. of fluid. Other forms were tested in shell vials or finger bowls containing 25–200 ml. of fluid. Of course, single series and replicates were with uniform volumes. Continuously flowing suspensions were not feasible; the more difficultly cultured marine animals were changed to fresh suspensions twice daily. For species that did not require frequent changing of the medium, subsequent assays with mosquito larvae or hermit crabs were commonly employed to verify the continued toxicity of test fluids.

Specimens were obtained from various sources. Protozoa were from laboratory cultures and were identified by Dr. D. H. Wenrich. Marine forms were collected at Woods Hole, mostly by the Supply Department, and the names used are those current at that laboratory. Other forms were collected or purchased, and identified by the authors.

Chemical tests for the presence of chitin were routinely performed on exoskeletons using the methods given by Campbell (1929). Positive results were obtained with Arthropods and the perisarc of Colenterates. The Bryozoan used (*Bugula*) gave atypical results suggesting a skeleton composed of something similar to, but not, chitin.[5]

For adsorption tests cuticle was removed from Horseshoe Crabs (*Limulus polyphemus*), manually cleaned of cells, rinsed thoroughly with distilled water, and dried. Part of this material was treated with 5 per cent NaOH at 100° C. until the color was removed and the pieces were negative to protein tests. This latter material is referred to herein as "chitin" although tests showed that a small percentage of the material had been changed to chitosan (see Campbell, 1929). Smaller amounts of cuticle were prepared from cockroaches and used in a few experiments, but most of the adsorption work was done with the more available cuticle of *Limulus*.

All tests were performed in replicate. Many of them were repeated.

RESULTS

The results obtained are summarized in the accompanying table. In all cases the toxin (DDT) was applied as a colloidal suspension in the bathing medium. The resistances or susceptibilities shown refer only to the indicated concentration in the surrounding medium. The figures are not comparable to median lethal doses and should not be interpreted as implying that "resistant" animals would be resistant to the injection of solutions or emulsions.

[5] The cuticle of *Bugula* is variously referred to in the literature as "chitinous" or "material akin to chitin." We applied the various chemical tests used by Campbell (1929) and others. The cuticle is not dissolved in hot concentrated KOH. After prolonged heating (which changed control pieces of known chitin to chitosan completely) it only crinkled in 3 per cent acetic acid instead of dissolving. The addition of one per cent H_2SO_4 gave no change and no precipitate. Another piece treated with $I + KI$ in water turned brown but only very slowly. After the addition of one per cent H_2SO_4 it remained brown for several minutes and then after 5–10 minutes slowly changed to a *greenish-brown* (instead of the clear violet given by chitosan). It dissolved immediately in 75 per cent H_2SO_4 but after standing no crystals resembling chitosan sulfate crystals were found. These tests suggest a similarity to chitin but certainly, at least in this species, the bryozoan cuticle is not true chitin.

TABLE I

The effective toxic concentrations of DDT for various animal phyla

All tests were replicated at least once. Susceptible species were tested repeatedly. Except where indicated otherwise tests were continued for 7 days or longer. The fluid on species that do not survive well in stagnant water was renewed daily or more frequently.

Group	Species	Temperature, C.	Type of water	Concentrations of DDT suspension					
				1:1,000,000,000	1:100,000,000	1:10,000,000	1:1,000,000	1:100,000	1:10,000
Protozoa	*Ameba proteus*	room	fresh	—	—	—	No effect (14 da)	Slow effect?	Slowly killed (3–10 da.)
	Paramecium sp.	room	fresh	—	—	No effect	No effect	No effect (1 mo.)	—
	Stylonychia putrina	room	fresh	—	—	—	No effect	—	No effect (1 mo.)
	Euglena gracilis	room	fresh	—	—	—	No effect	—	No effect (1 mo.)
	Chilomonas sp.	room	fresh	—	—	—	No effect	—	—
	Peranema sp.	room	fresh	—	—	—	No effect	—	No effect (1 mo.)
Coelenterata	*Obelia* sp.[1] *Campanularia* sp.[1]	15°	sea	—	Many killed	Killed (1–2 da.)	Killed (<1 da.)	—	—
	Tubularia sp.	15°	sea	—	No effect	Affected slightly [2]	Affected [2]	—	—
	Pennaria sp.	15°	sea	—	No effect	Killed slowly	Killed	Killed	—
	Hydractinia sp.	15°	sea	—	No effect	No effect	No effect	Affected [3]	—
	Hydra fusca	15°	fresh	—	No effect	No effect	No effect	Affected [3]	—
	Astrangia sp.	15°	sea	—	—	No effect	No effect	Affected [3]	—
Platyhelminthes	*Phagocata* sp.	room	fresh	—	—	No effect (1 mo.)	Killed (7–40 da.)	Killed (7–20 da.)	Killed (1–6 da.)
Nematoda	*Anguillula aceti*	room	fresh	—	No effect	No effect	No effect	No effect	No effect (1 mo.)
	Ascaris lumbricoides	35°	alkaline saline	—	—	—	No effect	No effect	No effect
Rotifera	*Philodina* sp.	room	fresh	—	No effect	No effect	No effect	No effect (1 mo.)	No effect
	Diplax sp.	room	fresh	—	—	—	No effect	No effect (1 mo.)	No effect
	Monostyla sp.	room	fresh	—	—	—	Some killed slowly (1–2 mo.)	Killed slowly (10–30 da.)	Killed slowly (7–10 da.)
Gastrotricha	*Chaetonotus* sp.[4]	room	fresh	—	—	No effect	No effect	—	—
Bryozoa	*Bugula* sp.	15°	sea	—	No effect (10 da.)	Killed slowly (4–6 da.)	Killed slowly (3–4 da.)	—	—

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[1] At least two, probably three, species of *Obelia* and *Campanularia* were used in these tests but identification of species in this group is quite difficult.

[2] *Tubularia* stems selected by Dr. L. G. Barth were set-up as in regeneration studies. All specimens regenerated but polyps quickly died in stronger concentrations and almost without exception did not regenerate a second time. Controls regenerated repeatedly.

[3] At 1:100,000 the specimens of *Hydra*, *Hydractinia*, and *Astrangia* were rounded and slow in reacting at the end of six days but were still living.

[4] These gastrotrichs were not tested separately. They were present in one of the rotifer cultures, and data cover only those test dishes, in which they were found.

TABLE I—*Continued*

Group	Species	Temperature, C.	Type of water	Concentrations of DDT suspension					
				1:1,000,000,000	1:100,000,000	1:10,000,000	1:1,000,000	1:100,000	1:10,000
Mollusca	*Littorina* sp.	room	sea	—	No effect	No effect	No effect	No effect	Killed slowly (6–7 da.)
	Mytilus sp.[5]	15°	sea	—	—	—	No effect	—	—
	Helisoma trivolva	25°	fresh	—	—	—	—	No effect	No effect
	Physa sp.	25°	fresh	—	—	—	—	No effect	Killed slowly
Echinodermata	*Thyone* sp.	room	sea	—	—	No effect	No effect	No effect	No effect
	Asterias sp.	room	sea	—	—	No effect	No effect	Killed (4 da.)	Killed (2 da.)
Annelida	*Aeolosoma* sp.	room	fresh	—	—	No effect	No effect?	Effect?[8]	—
	Polydora sp.[5]	15°	sea	—	—	—	—	No effect (7 da.)	—
	Nereis virens	15°	sea	—	No effect	No effect	No effect	Killed (4 da.)	Killed (2–3 da.)
	Lumbricus terrestris	15°	fresh[7]	—	—	—	—	No effect	No effect
Crustacea	*Artemia salina*	15°	sea	Most killed 2–6 da.	Killed (2–6 da.)	Killed (1–5 da.)	Killed (1–5 da.)	Killed (2–5 da.)	Killed (1–5 da.)
	Gammarus sp.	15°	fresh	Most killed (1–6 da.)	Killed (1–3 da.)	Killed (1–3 da.)	—	—	—
		28°	fresh	<half-killed (6 da.)	Killed (1–3 da.)	Killed (1–2 da.)	Killed (1 da.)	Killed (<1 da.)	Killed (<1 da.)
	Daphnia sp.	15°	fresh	Effect?[8]	Most killed (2–7 da.)	Killed (1–5 da.)	—	—	—
	Cyclops sp.	room	fresh	—	No effect	No effect?	Killed (<3 da.)	Killed (<3 da.)	Killed (<3 da.)
	Pagurus sp.	15°	sea	—	—	Killed (1–3 da.)	Killed (1 da.)	—	—
	Emerita talpoida	15°	sea	—	—	Effect?	Killed (1–2 da.)	Killed (1–2 da.)	—
Insecta	*Aedes aegypti* larvae	15°	fresh	Half killed (7 da.)	Killed (2–7 da.)	Killed (2–4 da.)	Killed (2–4 da.)	—	—
		28°	fresh	No effect (7 da.)	Most killed (7 da.)	Killed (1–4 da.)	Killed (1–2 da.)	—	—
	Culex pipiens larvae	15°	fresh	—	—	Killed (<1 da.)	—	—	—
		30°	fresh	—	—	Killed (1–3 da.)	—	—	—
	Chironomus sp. larvae	room	fresh	—	—	Killed (1–2 da.)	—	—	—
	Chaoborus sp. larvae[9]	10°	fresh	Some killed (5 da.)	Killed (5 da.)	Killed	—	—	—
		30°	fresh	No effect (5 da.)	Most killed (5 da.)	Killed	—	—	—
Fungi	*Rhizopus nigricans*	room	fresh	—	—	—	—	—	No effect
	Penicillium sp.	room	fresh	—	—	—	—	—	No effect
	Saccharomyces cervisiae	room	fresh	—	—	—	No effect	No effect	—

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[5] Only a few specimens (not a statistically significant number) were tested of *Mytilus* and *Polydora*. The negative results suggest that these are both resistant species, as are other members of their phyla.

[6] At 1:100,000 there was an initial decrease in numbers followed by an increase. It would seem that some are killed but that the survivors reproduce readily.

[7] The earthworms were tested in flat dishes with the fluid not sufficiently deep to cover the animals completely.

[8] Anderson (1945) records an effect on *Daphnia* at concentrations greater than one part per billion and no effect at concentrations lower than one part per billion. Apparently his cultures were slightly more susceptible to DDT than ours.

[9] Experiments on *Chaoborus* by Dr. Hsing Yun Fan.

Of the species tested, the Protozoa, Nematoda, Gastrotricha, Rotifera, Mollusca, Annelida, and Echinodermata were resistant to DDT suspensions or in the case of a few species, were slowly killed by very concentrated suspensions. The single species of the Platyhelminthes tested was comparatively resistant. The single species of Bryozoa was quite susceptible. And, the various species of Arthropoda and those Coelenterata with a complete perisarc were highly susceptible. The most interesting data come from the hydrozoan Coelenterata where those species with a complete chitinous perisarc (*Obelia* and *Campanularia*) are highly susceptible, those species with a chitinous perisarc over only the main stalk (*Tubularia* and *Pennaria*) were less susceptible, and those without a perisarc (*Hydra* and *Hydractinia*) were nearly resistant. The bryozoan, *Bugula*, is an intermediate which is almost as susceptible to DDT as the arthropods and sensitive colenterates. *Bugula* has a complete cuticle which is composed of some substance seemingly similar to but not identical with chitin.[5] Correlated with this similarity is the more or less similar relationship to DDT poisoning from dilute solutions. The most reasonable assumption at present is that the cuticle of *Bugula* behaves towards DDT similarly to chitinous cuticles but that it is less effective (or possibly the animals are really more resistant).

The data presented herein do not include vertebrates. The most nearly comparable data on fishes indicate an intermediate sensitivity (Eide, Deonier, and Burrell, 1945; Ginsburg, 1945) and that concentrations entirely adequate for mosquito control (i.e., well above the minimum lethal concentration) do not injure fish (Metcalf *et al.*, 1945). With terrestrial vertebrates truly comparable data are not available. The closest parallel is with aerosols and mists; very high concentrations of DDT applied in this manner cause little or no injury to mammals (Neal *et al.*, 1944). DDT in solution in oil can be absorbed through mammalian skin (Draize *et al.*, 1944) but the dosage needed to produce an effect is fairly high. Also, it is well known that DDT has been approved for use as a powder on vertebrates to kill ectoparasitic insects. The data, then, suggest that for external applications more or less comparable to our data, fish occupy an intermediate position in susceptibility—being some ten to one hundred times more resistant than mosquito larvae. Mammals seem to be much more resistant to external applications than either fish or insects.

The obvious correlation of the above data is to the chitinous exoskeleton. Actually there are a number of possibilities, notably (1) that some of the animal groups are truly more or even completely resistant to the toxin; (2) that certain animal groups may be able to detoxify or excrete DDT better than others; (3) that there may be a reaction between a chitinous cuticle and DDT to produce some other more toxic substance; or (4) that the susceptibility may be due solely to penetration and accumulation which is favored by a chitinous cuticle. The evidence at hand suggests that both the first and last named possibilities are partly correct. Considering the points in order:

Data derived from the feeding and injection of DDT solutions (not suspensions) and emulsions show that on a dosage/weight basis mammals have approximately the same order of susceptibility as cockroaches (Draize, *et al.*, 1944; Smith and Stohlman, 1945; Chadwick, 1945). While some variability is obtained among insects (Chadwick, 1945), the fact remains that internal applications do not show the tremendous differences between vertebrates and insects that external applications show.

However, there must be a considerable amount of variability in true resistance among different groups on invertebrates. *Ascaris* is immune to external applications of DDT suspensions but the median lethal dose for injection of emulsions (on a dosage/weight basis) seems at least several times as high as the MLD for cockroaches.[6] And, injection of DDT emulsions into the common aquarium snail, *Helisoma trivolva*, was apparently without effect even when one mg. of DDT was injected into a snail whose weight (without the shell) was less than a gram. Unfortunately, we have no precise knowledge of the site of injection but at least it was into the interior of the animal and sometimes into the tissues. It seems clear from these data (1) that there must be considerable difference among various animals as to the effect of a given amount of DDT after it is *in* the animal, and (2) that relative absorbability also varies.

The second possibility, detoxification or excretion by certain groups, seems unlikely both because of the similarity of vertebrate and insectan median lethal doses on injection and because of the great dissimilitary in excretory organs. The role of detoxification may actually be considerable. One non-toxic breakdown product, dichlorodiphenylacetic acid, has already been positively identified in mammalian urine (White and Sweeney, 1945). Detoxification, however, does not seem to contain the full answer to the wide variations in susceptibility reported in this paper.

The third possibility, a new and more potent toxin arising from a reaction with chitinous cuticles, seems eliminated by several points. The above mentioned similarity of median lethal doses and symptoms following injection of emulsions is against it. Also pertinent is the fact that DDT suspensions have no visible effect on cultured tissues whether chitin particles are added to the medium or not (Lewis and Richards, 1945).

The above considerations led to two different types of tests on the fourth possibility; namely, that cuticles might facilitate the entry of DDT into animals. If chitinous cuticles facilitate the entry of DDT it might be possible to find aquatic invertebrates which are immune to external applications but killed by injections. We have data on one such case. *Acaris* does not have a chitinous cuticle and is completely resistant to immersion in the strongest DDT suspensions. It is killed by the injection of emulsions although requiring a somewhat higher dose than cockroaches and vertebrates.

While these data on *Ascaris* are suggestive, they, of course, do not prove anything about chitinous cuticles. More direct data were sought by trying to find a possible mechanism by means of which chitinous cuticles might facilitate the entry of DDT into an arthropod or coelenterate. The following data suggest that such a mechanism does, indeed, exist and that adsorption by chitin plays an important role in it.

Obviously, DDT can penetrate insect cuticles since external applications can kill even when the possibility of ingestion is eliminated. Tests showed that DDT could be adsorbed from water by activated charcoal. DDT suspensions in water lost all toxicity to mosquito larvae after being stirred with charcoal and filtered. It follows from this that the DDT particles have been removed by the charcoal. Similarly, we found that DDT could be adsorbed from aqueous suspensions by arthropod cuticle and even better by purified chitin. The results are only qualitative; accordingly, tabular data are not presented. Cuticle and purified chitin from *Limulus* and from cockroaches were prepared as described in the section on methods. An excess of either material was added to a moderately dilute suspension of DDT in distilled water, equilibrated at 6° C., and filtered. The filtrate had its expected toxicity to mosquito larvae greatly reduced or abolished. From this bio-assay it follows that the cuticle or chitin had removed part or all of the DDT. Cuticle or chitin was then added to a more concentrated suspension, equilibrated at 6° C., filtered, and the pieces of cuticle or chitin were soaked twice in cold distilled water and filtered to wash off any DDT that might be present in the water on the surface or within the matrix of the pieces. The lots of cuticle and chitin were then soaked in hot distilled water (90° C.) adjusted to pH 3.8 with 0.1 molar H_2SO_4, filtered, and the filtrate tested by the usual bio-assay method with mosquito larvae. Symptoms and mortality showed that DDT had been recovered. Since DDT could be removed and recovered by these adsorption methods it follows that cuticle and purified chitin are capable of adsorbing DDT.

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[6] We determined the median lethal dose for *Ascaris* only to a rough order of magnitude and so prefer not to cite a definite quantitative figure.

In addition to the controls accompanying the above adsorption tests, a number of other things were tested. As judged by subsequent bio-assay a small amount of DDT was adsorbed by cleaned dry spines of sea urchins, but none was adsorbed by snail shells, coral, a suspension of erythrocytes (cow), or chunks of muscle (*Limulus*).

Another point which is consistent with the idea that adsorption by chitin facilitates the entry of DDT, is that mosquito eggs are resistant to any concentration of DDT tested, whereas, the larvae on hatching are susceptible. This might represent differences in embryonic stages or a barrier between the egg shell and the enclosed embryo or larva but it could conceivably be due to the fact that larvae have a chitinous cuticle, whereas, the insect egg shell contains no chitin.

If adsorption phenomena are playing an important role, one would expect to find a negative temperature coefficient, i.e., that DDT is more toxic at lower temperatures than at higher temperatures. Solubility tests show that DDT has a positive temperature coefficient for solubility in both polar and apolar solvents. Accordingly, a negative temperature coefficient is good evidence in support of an adsorption hypothesis. Actually the temperature relationships are complex and not fully understood. Data obtained to date show that at low concentrations a negative temperature coefficient is, indeed, obtained but that at higher concentrations this changes to a positive temperature coefficient. However, it seems logical that a concentrating mechanism would be most dominant at lower concentrations. Accordingly, the temperature data support the hypothesis that adsorption of DDT by chitinous cuticles acts as a concentrating mechanism. The shift to a positive temperature coefficient at higher concentrations would seem to imply that other factors are involved and that the adsorption by chitinous cuticles is only for concentrating the toxin, the actual toxic action of DDT being something else.

Most of the temperature studies have been performed using mosquito larvae at 15° and 28° C., but similar results have been obtained with the fresh water shrimp

Gammarus.[7] Lindquist *et al.,* (1945) have independently found a negative temperature coefficient in spray work with adult flies, and during the past year it has come to be generally considered that DDT works better in insect control operations in cool climates than in hot climates. Attempts to obtain data with coelenterates at high and low temperatures failed because we were not successful in keeping the species available at Woods Hole alive in warm, non-running water.

Discussion

From the data presented here, we conclude that the sensitivity of certain animal groups to DDT applied externally as a colloidal suspension in the bathing medium is correlated with the presence of a chitinous or similar cuticle in addition to true differences in tissue susceptibility. This correlation is supported by the similarity of toxicity to certain animals when emulsions are injected in contrast to dissimilarity from external applications, and by direct demonstration of adsorption of DDT by chitin and chitinous cuticles, and non-adsorption by certain other types of exoskeletons and tissues. The idea that adsorption processes are important is further supported by the demonstration of a negative temperature coefficient for toxicity at lower concentrations. The data seem to concern only the differences in sensitivity and not to explain the toxic action itself. The exceptions recorded herein (e.g., snails) show that the action of DDT is more complex but they do not invalidate the cuticle relationship. Accordingly, we propose as a hypothesis that chitinous cuticles act to selectively concentrate DDT from the surrounding medium by adsorption processes.

This hypothesis is in some respects similar to the old idea of penetration due to partition coefficients. Several authors have invoked the idea of partition coefficients to explain insecticide penetration (Wigglesworth, 1941; Richards and Weygandt, 1945). The present hypothesis for concentrating DDT varies in substituting an active adsorbing interface of chitinous cuticle. On theoretical grounds Hurst (1943) has suggested that the insect cuticle may adsorb toxins, as work by Castle (1936) had already indicated. The data in the present paper, however, give the first direct demonstration that a chitinous cuticle can adsorb an insecticide and that the adsorption may play a role in selective toxicity to different groups of animals.

Since one is inclined to think of the arthropodan cuticle as a solid sheet of material, it is natural to ask where sufficient surface is available for the demonstrated adsorption. A possible clue is given by Castle's (1936) work on the "oriented imbedding" of organic substances in chitin. This is a kind of adsorption process that apparently utilizes the surfaces of known intermicellar spaces. Perhaps the same intermicellar surfaces may be available for the adsorption of DDT.

Obviously, adsorption by the cuticle can be only part of the story of the action of DDT. Some mechanism is needed to transport the DDT from the cuticle to its effective locus inside the animal. We have no direct evidence as to what this mechanism is. Perhaps one can interpret the data from distribution of the radioactive bromine analog (Hansen, Hansen, and Craig, 1944) to mean that DDT is more soluble inside insects than in water. Such a difference in solubility in connection with an actively adsorbing cuticle could account for a selective accumulation in insects and other groups with similar cuticles.

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[7] More recently Dr. Hsing Yun Fan has made an intensive study of the negative temperature coefficient using both mosquito larvae and midge larvae (*Chaoborus* sp.). His data will be published subsequently.

A careful consideration of reported exceptions is necessary. Many papers have been published on the use of DDT as an insecticide.[2] These papers, while showing a general susceptibility of insects to DDT, are mostly not comparable to the present paper. They are not primarily concerned with the resistance or susceptibility of the various species but with the practicability of controlling pest insects under natural conditions. A susceptible insect that is not reached by the insecticide in the field will not be killed. Also the thickness, dryness, nature of the epicuticle (external layer outside of chitinous cuticle), and other properties of the cuticle vary greatly from one group to another within the arthropods (Richards, 1944). One should expect variation in susceptibility with these variables. In some cases it seems clear that forms which are not satisfactorily controlled are nevertheless susceptible. For instance ticks and mites are said not to be well controlled by DDT (Cox, 1945) yet they are by no means resistant (e.g., Gouck and Smith, 1944; Smith and Gouck, 1944; Vargas and Iris, 1944, etc.), and at least one species (*Rhipicephalus sanguineus*) is readily controlled by DDT. In some other cases, notably certain beetles with thick, hard, dry cuticles, insects seem little or not at all affected. Since these are terrestrial species, the difficulty in obtaining data comparable to data presented in this paper is considerable. In the Crustacea there is a considerable range of variability; most species tested were highly susceptible but the copepods are much less susceptible (see also Anderson, 1945; Seagren, Smith, and Young, 1945). It seems certain that more and less resistant species do occur among arthropods even though available data refer principally to practical field tests. While it is possible to offer hypothetical explanations of these variations on the basis of differences in cuticle structure, variations in degree of effect seem to be a factor also (Chadwick, 1945). However, the known susceptibility of arthopods in general is so broad that we feel inclined to suggest that the variations will be explained without vitiating the hypothesis advanced in the present paper.

The fungi with chitinous cell walls (*Rhizopus,* etc.) are a true exception. They are apparently indifferent to DDT in the medium bathing them. This resistance cannot be evaluated until we are more certain as to the method by which DDT kills. One obvious possibility lies in the differences between plants and animals, particularly, the absence of a nervous system in plants.

Another line of exceptions concern the intermediate susceptibility of fishes (Eide, Deonier, and Burrell, 1945; Ginsburg, 1945, and others). No explanation of this is offered, but there is no reason to assume that intermediation of a chitinous cuticle is the only means by which DDT can enter an aquatic animal.

The most serious exceptions, however, are those invertebrates which are not affected by the injection of emulsions (snails). These are species representative of a group unaffected by external applications. No explanation of this exceptional lack of effect on injection is offered in the present paper, but obviously the snails demonstrate beyond question that more is involved in the whole story of DDT action than just the facilitation of entrance by an appropriate cuticle.

Summary

1. Throughout the animal phyla there is a correlation between the presence of a chitinous cuticle and susceptibility to external applications of DDT. Those aquatic animals with a chitinous cuticle (arthropods and certain coelenterates) are highly sensitive to external applications of DDT, other animals are not so susceptible although there is considerable variability.

2. The correlation to a chitinous cuticle is supported by studies on various coelenterates, on adsorption, and on temperature coefficients. Coelenterata with respectively a complete, partial, and no chitinous perisarc are respectively highly sensitive, somewhat sensitive, and nearly insensitive to DDT. DDT can be adsorbed by chitin and chitinous cuticles and at low concentrations shows a negative temperature coefficient for toxicity to arthropods.

3. From these data a hypothesis is proposed that chitinous cuticles facilitate the entry of DDT into the animal body by selectively concentrating the compound by adsorption phenomena.

4. This is the first demonstration that an insecticide can be adsorbed by chitinous cuticles and the first direct evidence that such adsorption actually can play a role in insecticide action.

5. The present paper does not consider the nature of the toxicity of DDT to protoplasm. The data given here refer only to the selective action of DDT as a function of penetration facilitated by chitinous exoskeletons. The shift to a positive temperature coefficient at higher concentrations, the lack of effect from injecting DDT emulsions into snails, and the variability in median lethal doses for injected DDT emulsions in different animals, all indicate that the selective adsorption of DDT by chitinous cuticles is only a part of the story of the toxic action of this compound.

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PROTOPLASMIC VISCOSITY CHANGES IN DIFFERENT REGIONS OF THE GRASSHOPPER NEUROBLAST DURING MITOSIS

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Protoplasmic viscosity has been studied by a variety of methods. Each has certain advantages and certain limitations, with regard to the kind of living material to which it is best suited and the accuracy of the results that it will give. These methods and their uses have been reviewed critically by Heilbrunn (1928, 1943) and therefore will not be considered in detail here. Of the several that have been employed the brownian movement method is probably best suited to viscosity studies involving limited regions of the single cell. It may be applied in either of two ways: 1) to calculate the absolute viscosity by measuring the displacement in one direction of the molecularly-bombarded particle and the time required to bring this about, certain other characteristics of the cell and its environment being known, or 2) to compare the viscosities of different regions of a cell at different mitotic stages or under different experimental conditions through observations of the relative speeds of brownian movement. The latter has been used in the present investigation.

The neuroblasts of the grasshopper embryo possess several advantages in such a study. Since they are situated on the ventral side of the embryo, they can be brought next to the cover glass in hanging-drop preparations and microscopic observations of the mitotically active, living cells can extend over several hours. All the cell features that are visible in the usual fixed and stained preparation can be seen, and many phases of the mitotic cycle can be identified readily. The cell is relatively large, measuring about 30 μ in diameter. It maintains a visible polarity from one mitotic division to the next, so that a given region can be located in any cell at any stage of division. The cytosome contains large numbers of mitochrondria, which not infrequently find their way into the spindle during mitosis. By observing the brownian movement of these tiny bodies it is possible to compare the viscosities of the surrounding protoplasm of all non-nuclear parts of the cell at all stages of the mitotic cycle.

MATERIAL AND METHODS

Embryos of *Chortophaga viridifasciata* (De Geer) were prepared by the hanging-drop method previously described for this material (Carlson and Hollaender,

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[1] The preliminary observations on which this study is based were made as Rockefeller Fellow in the Natural Sciences at the Genetics Laboratory of the University of Missouri in the winter of 1940–41 and at the Biological Laboratory, Cold Spring Harbor, in the summer of 1941. The work has been completed with the aid of a 1944–45 grant from the University Research Committee of the University of Alabama.

1944). The culture medium, which must be isotonic with the embryonic cells, is made up as follows:

NaCl	0.70	gm.
KCl	0.02	gm.
$CaCl_2$	0.02	gm.
$MgCl_2$	0.01	gm.
NaH_2PO_4	0.02	gm.
$NaHCO_3$	0.005	gm.
Glucose	0.80	gm.
H_2O (pyrex redistilled)	100.0	cc.

The pH of this solution is approximately 6.5, which is about the pH of the grasshopper egg yolk. A small amount of yolk is added to each hanging-drop to provide nitrogenous food materials for the cells.

Preparations were studied in a constant temperature box enclosing all of the microscope except the upper part of the body tube and arm. All observations were made at 26 ± 0.5° C. The light used as a source of microscope illumination was passed through copper sulfate solution to filter out the heat.

Observations were made exclusively on neuroblasts. These cells are shown in representative mitotic stages in Figure 1, which is based on camera lucida sketches of living cells. It will be noted that the neuroblast deviates from the typical cell in two main respects. First, the nucleus is not spherical but roughly hemispherical with a central cytoplasmic core connecting the polar and apolar regions of the cell (Figs. 1*A* and 1*B*). Second, the cell divides unequally to form a small daughter ganglion cell and a large daughter neuroblast (Fig. 1*J*.) The great advantage of these cells is their large size, which makes it possible to observe and study in the living, unstained cell many structural features that are very difficult or even impossible to deal with in smaller cells.

The mitochondria of the neuroblast are spheroidal in shape and measure approximately 0.3–0.5 μ in diameter.

TERMINOLOGY

Relation of viscosity to brownian movement.

The relation between the rate of brownian movement and viscosity is given by Einstein's equation

$$D_x^2 = \frac{RTt}{N3\pi\eta a}$$

in which D_x is the displacement of the particle along the x axis; *R*, the gas constant; *T*, the absolute temperature; *t*, the time; *N*, the Avogadro number; *a*, the radius of the particle; and η the viscosity expressed in poises. Accordingly, the viscosity of the dispersion medium varies inversely as the square of the displacement of a given particle along one axis.

The rapidity of brownian movement of the mitochondria in selected regions of the cell was observed and classified according to the following system. The

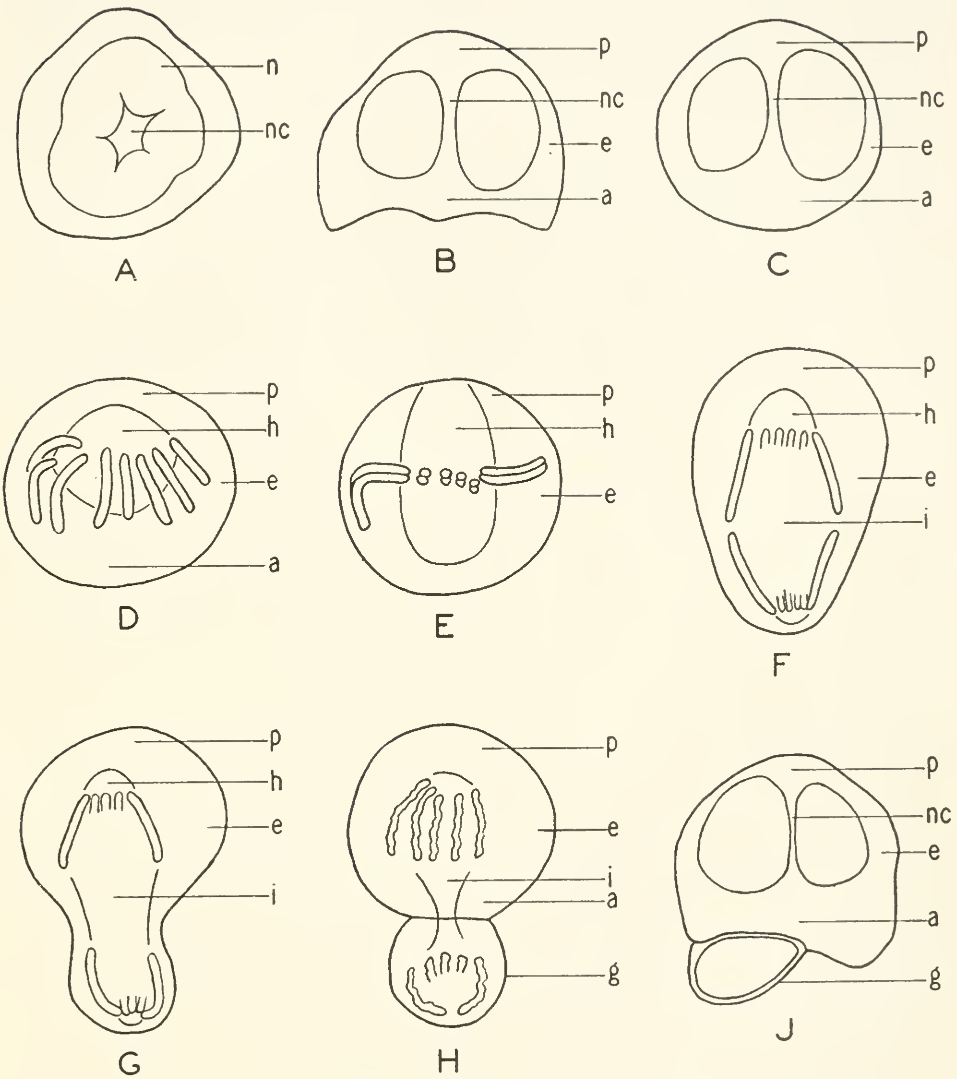

FIGURE 1. Representative stages in the mitotic cycle of the grasshopper neuroblast, reconstructed from camera lucida sketches of living cells growing in culture medium. *A* and *B*, polar and side views, respectively, of interphase or prophase cells; *C*, late prophase a few minutes before breakdown of the nuclear membrane, the cell having acquired a spherical form; *D*, prometaphase; *E*, metaphase; *F*, middle anaphase; *G*, late anaphase; *H*, early telophase; *J*, late telophase, the cell having lost its spherical form. *a*, polar cytoplasm; *e*, equatorial cytoplasm; *g*, ganglion cell; *h*, half spindle; *i*, interzonal region; *n*, nucleus; *nc*, nuclear core cytoplasm; *p*, polar cytoplasm.

viscosity values and the relative speeds of brownian movement are designated as follows:

Very high: No visible movement of mitochondria.

High: Movements very limited, discernible only with prolonged observation.

Medium: Movements readily observable, but not rapid.

Low: Movements quite free and rapid, but individual mitochondria can be followed with the eye.

Very low: Movements so rapid that individual mitochondria cannot be followed with the eye.

Even if the ability to make purely objective distinctions between adjacent members of this series be open to question, values separated by one class, viz., very high and medium, high and low, or medium and very low, can be distinguished readily and would give results essentially similar to those obtained.

Mitotic stages

The stages for which data were obtained and their distinguishing characteristics in the living, untreated state, when observed through 12.5 × compensating oculars and a 2 mm. oil immersion objective, are:

Interphase: Nuclear threads appear in optical section like small, rounded, scattered granules in an otherwise homogeneous nuclear background. Period of growth.

Very early prophase: Nuclear granules smaller in size. Threads have begun to appear in the previously homogeneous background.

Early prophase: Nucleus filled with extremely fine chromatin threads. No nuclear granules.

Middle prophase: Chromatin threads of appreciable thickness. Ends when about seven chromosomes in cross-section can be counted in one-fourth the nuclear circumference.

Late prophase: Chromosomes well-formed. Toward the end of this period cell assumes spherical shape (Fig. 1*B*, 1*C*). Ends with the simultaneous disappearance of the nuclear membrane and appearance of the spindle.

Prometaphase (Fig. 1*D*): Chromosomes straighten and move into the equatorial plane. Spindle develops.

Metaphase (Fig. 1*E*): Chromosomes in equatorial plane.

Early anaphase: Begins with the initial separation of the chromatids and ends when the distal ends of the chromosomes leave the cell equator.

Middle anaphase (Fig. 1*F*): This stage is terminated as the cleavage furrow penetrates to the interzonal region.

Late anaphase (Fig. 1*G*): Ends as the chromosomes lose their sharp outlines and the cleavage furrow appears to be complete.

Early telophase (Fig. 1*H*): This stage lasts until the nucleoli become visible.

Middle telophase: The nucleoli increase in size while retaining their spherical shape, the nuclear membrane re-forms, the interzonal spindle remnant disappears, and the cell re-assumes the hemispherical form.

Late telophase (Fig. 1*J*): Begins as the outlines of the nucleoli become irregular. Ends as linear arrangement of chromatin granules is lost.

The relative duration of these stages is given in an earlier paper (Carlson, 1941).

Regions of cell

Data have been obtained for six different regions of the cell, which are labeled in representative stages of the mitotic cycle in Figure 1.

Polar cytoplasm: This includes the region about the spindle pole of the daughter neuroblast when a spindle is present. At other stages it is the region within which the spindle pole of the daughter neuroblast will develop preparatory to the next division.

Apolar cytoplasm: The cytoplasm situated near the place of formation of the preceding cleavage furrow. It is opposite the polar region.

Equatorial cytoplasm: This is the portion of the cell that is situated at or near the cell equator in metaphase and anaphase and midway between the polar and apolar regions during the remainder of the mitotic cycle.

Nuclear core cytoplasm: The cytoplasmic core that passes through the nucleus (see p. 110).

Half spindle: This includes the developing spindle at prometaphase, the fully formed spindle at metaphase, and at anaphase the portion of the spindle lying between the pole and the plane in which the kinetochores of the adjacent chromosome group are situated.

Interzonal region: This is the part of the cell lying inside the ring of interzonal fibers and situated between the two separating groups of daughter chromosomes. It makes its appearance as the chromatids separate at early anaphase and persists as a spindle remnant into middle telophase.

Nucleus: No information that seemed reliable was secured for this part of the cell. Tiny nuclear granules comparable in size to the mitochondria showed very limited movement at various stages of the mitotic cycle. This might indicate a persistently high viscosity or it might mean merely that these granules are attached to the chromatin threads, which form a semi-rigid framework within the nucleus, and would therefore not be expected to exhibit much brownian movement. Bělař (1929a), however, has reported a rapid brownian movement of tiny granules of unknown nature situated in the ground substance of the nucleus of the grasshopper spermatocyte during prophase and telophase, indicating, according to him, that this substance is quite fluid.

OBSERVATIONS

The curves shown in Figure 2 were constructed in the following way. A linear series of arbitrary numerical values was assigned to the five selected viscosity classes described previously. Four observations were made for each region of the cell on different days and with different preparations. These were averaged and plotted. Because the determination of each of these was to a certain extent subjective, in that it depended on the judgment of the observer, and because the vis-

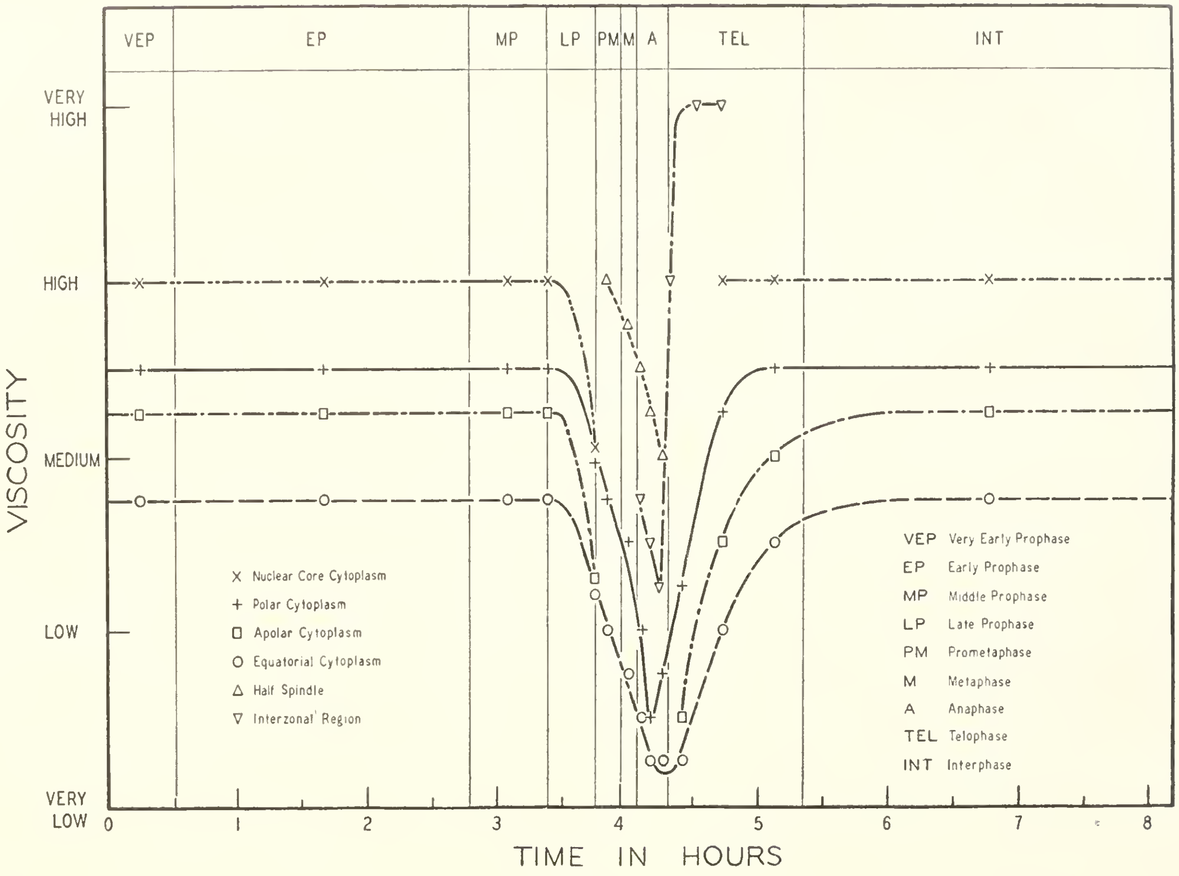
VEP
EP
MP
LP
PM
M
A
TEL
INT
VERY HIGH
HIGH
MEDIUM
LOW
VERY LOW
VISCOSITY
0
1
2
3
4
5
6
7
8
TIME IN HOURS
X Nuclear Core Cytoplasm
+ Polar Cytoplasm
□ Apolar Cytoplasm
O Equatorial Cytoplasm
△ Half Spindle
▽ Interzonal Region
VEP Very Early Prophase
EP Early Prophase
MP Middle Prophase
LP Late Prophase
PM Prometaphase
M Metaphase
A Anaphase
TEL Telophase
INT Interphase

FIGURE 2

cosity varied slightly from preparation to preparation and from cell to cell, certain of these curves showed irregularities. In order to discover whether these were the result of experimental error or of actual viscosity shifts, new cultures were prepared, checked, and any necessary corrections made. It is believed that the graph in its present form represents quite accurately the cycle of viscosity changes in the different regions of the cell during mitosis. It should be emphasized, however, that the five arbitrarily-chosen viscosity values represented by ordinates are not necessarily a linear series of absolute values, as their graphical treatment implies.

During the greater portions of interphase and prophase the viscosity of the cytosome maintains a relatively high and constant value. In late prophase, a few minutes before the disappearance of the nuclear membrane, a rapid fall in viscosity is initiated. This change begins as the cell changes in shape from hemispherical (Fig. 1*B*) to spherical (Fig. 1*C*). The increase in the rapidity of mitochondrial movement is distinguishable first at the juncture of the equatorial and apolar regions as this part of the cell begins to draw in coincidentally with the start of the rounding-up process. From this region the lowered viscosity spreads into the apolar region, which thickens as the rounding-up proceeds, and subsequently into the equatorial and polar regions. Obviously, some of this is due to the actual movement of the cytoplasm, which must of necessity shift in position as the cell shape changes, but this can hardly be responsible for more than a small fraction of the viscosity change that takes place at this time. Since this alteration in shape occupies only about 5–10 minutes, this sequence of changes does not appear in the graph. The viscosity continues to fall through prometaphase and metaphase to reach a minimum in anaphase. The progress of the cell through telophase is accompanied by a steady rise in viscosity until the interphase-prophase level is attained. During telophase the hemispherical form of the cell is re-assumed (Fig. 1*J*).

The half spindle appears to show a slight fall in viscosity from prometaphase to early telophase, by which time it is too small for further study.

As the chromosome halves begin to separate at early anaphase, the interzonal region between the groups often contains one or more mitochondria showing a moderate amount of brownian movement. During middle and late anaphase large numbers of mitochondria move into the interzonal region, until they are as concentrated as outside, except for a small region inside each of the separating chromosome groups. Their movement is quite rapid, but less so than that of the mitochondria outside in the equatorial region. As the cleavage furrow presses in against the interzonal fibers, the transverse diameter of the interzonal region decreases and the motion of the mitochondria quickly slows down and then stops entirely. The fact that these stationary bodies are lined up in rows parallel with the long axis of the spindle makes it appear as if they had been caught among interzonal fibers of higher viscosity as these were pressed inward by the deepening cleavage furrow. By late telophase these fibers are no longer visible.

The high viscosity level of the nuclear core cytoplasm immediately after the formation of the nucleus and core may be due to the fact that this is the region occupied earlier by the spindle remnant of the preceding division.

Discussion

The brownian movement method

Of the three most frequently used methods of making determinations of protoplasmic viscosity changes, viz., centrifuge, microdissection, and brownian movement, the last has the advantage of producing no physical disorientation of the cell contents. It can be depended on to give reliable results, however, only if certain factors are taken into consideration:

1) The protoplasm surrounding the granules in brownian movement must be homogeneous, if the viscosity of the protoplasm as a whole, exclusive of the granules, is being determined. If regions of different viscosity are present, observations of the rate of brownian movement of a particle will indicate only the viscosity of the material immediately surrounding it. The polar, equatorial, apolar, and nuclear core regions of the neuroblast cytosome are visibly homogeneous except for the mitochondria, and the fact that these bodies appear to migrate about and pass one another in the course of their shorter zig-zag movements demonstrates that they are not enclosed in a substance of one viscosity that is in turn surrounded by material of another viscosity. The spindle, however, is probably an exception to this (see p. 118).

2) Alterations in the size of the mitochondria from one stage of mitosis to another would cause an apparent shift in the viscosity of the surrounding medium, even though no actual change took place. Such changes could conceivably result either from shrinking and swelling as a consequence of osmotic shifts or from division and growth. The latter can be ruled out in the case of the neuroblast because very few mitochondria would need to divide in each cell generation to make up for the loss to the daughter ganglion cell, which receives very little cytoplasm and very few mitochondria. The resulting effect on brownian movement would be insufficient to alter the general viscosity picture. With regard to osmotic changes, Lewis and Lewis (1915) report that immersing cells in hypotonic solution causes swelling of the mitochondria. Bělař (1929a), on the other hand, states that mitochondria are particularly insensitive to the usual swelling effects of hypotonic solution. Unfortunately, the neuroblast mitochondria are so small and move so rapidly at certain stages that exact comparisons of size by actual measurements are impossible. Careful visual comparisons of the mitochondria of adjoining cells in different mitotic stages, however, have failed to reveal any significant size differences. As far as the present study is concerned, swelling of the mitochondria of late prophase, prometaphase, and metaphase cells by intake of water (p. 119) would tend to reduce rather than augment the observed viscosity shift in these stages; therefore, the actual change would be even greater than that shown in Figure 2.

3) If the granules under observation are so crowded as to interfere with each other's movements, the viscosity values obtained at the lower levels will be too high; for frequent collisions will retard their movements. The mitochondria of the neuroblast are definitely crowded, and this could easily result in appreciable errors in absolute viscosity values based on the migration of a given body a certain distance in a certain time, according to the method described by Pekarak (1930). Much of this error is doubtless avoided in the present study, however, because viscosity determinations were based on the rapidity with which the particles as a group dance about rather than on the total distance they migrate in a given interval of time.

Results of related studies

Of the several studies made on viscosity changes of the whole cytoplasm during mitosis, the centrifuge determinations of Heilbrunn (1917, 1921) for the marine invertebrate egg (Arbacia, Cumingia, and Nereis) and of Kostoff (1930) for meiotic cells of Nicotiana and the brownian movement determinations of Kato (1933) for the meiotic cells and staminate hairs of Tradescantia have given results that are similar to those I have obtained for the polar, apolar, and equatorial cytoplasm of the grasshopper neuroblast. All these studies show that the viscosity is relatively high during interphase, when interphase is referred to at all, high during all or most of prophase, falling during metaphase, lowest at anaphase, and rising at telophase.[2] The similarities in these results are all the more striking in view of the great diversity of the cell types used. The marine invertebrate egg contains relatively large quantities of ergastic matter, such as oil globules and yolk and pigment granules, and develops during mitosis an astral system and spindle that involve a large portion of the protoplast. The pollen mother cell, though consisting of a more or less homogeneous cytosome with relatively small spindle and no astral system, nevertheless is atypical mitotically because of the extended prophase and the two successive meiotic divisions without an intervening interphase. The staminate hairs of the plant contain large vacuoles with only a peripheral film and a few central strands of cytoplasm. The grasshopper neuroblast contains a fairly homogeneous cytosome with no visible ergastic matter or vacuoles but an abundance of mitochondria. The spindle is moderate in size and no asters are visible. This suggests strongly that viscosity change is a fundamental factor in mitosis, and is largely independent of individual pecularities of the type of cell studied.

Zimmermann (1923), who studied the viscosity of the alga, Sphacelaria, by means of the centrifuge and brownian movement, and Seifriz (1920), whose studies of the marine invertebrate egg are based on microdissection, place the viscosity fall and minimum somewhat in advance of this mitotically. According to Zimmermann the viscosity is lowest at metaphase, while Seifriz finds it lowest during late prophase and metaphase. Both describe the viscosity as rising at anaphase and high at telophase.

In contrast with these findings are the results obtained by Chambers (1917, 1919) from studies of Arbacia and Echinarachnius eggs with the microdissection needle. He reported that the viscosity of the greater part of the cell is low during prophase, rising during metaphase, high during anaphase and early telophase, and falling in late telophase. He related the viscosity to the state of development of the amphiaster: low when no amphiaster is present, rising as the amphiaster forms, highest when it has reached its maximum development, and falling as the amphiaster disappears at the end of mitosis. He does, however, describe a liquefaction of the equatorial region previous to anaphase and persisting through cleavage.

Fry and Parkes (1934) duplicated as closely as possible the centrifuge studies of Heilbrunn on Arbacia, Cumingia, and Nereis eggs. The results they obtained for viscosity in relation to time after fertilization were identical with those reported earlier by Heilbrunn; in fact, in their paper they used Heilbrunn's curves for Arbacia and Nereis eggs. They interpreted this data, however, as supporting

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[2] Heilbrunn associates the pre-cleavage fall and the post-cleavage rise in viscosity of the Arbacia egg with spindle development and disappearance, respectively.

Chambers' conclusions, claiming that Heilbrunn had misidentified certain of the mitotic stages. This would place viscosity changes of the marine invertebrate egg in entire disagreement with those of the grasshopper neuroblast, in which there can be no question of the correct identification of the different mitotic stages.

Kostoff's description of viscosity changes in somatic cells of Nicotiana (1930) is not in accord with any of these results. Using the centrifuge technique, he reported two cycles of viscosity change for each mitotic cycle: high viscosity at prophase and anaphase, and low viscosity at metaphase and interphase.

Observations on the viscosity of the spindle are complicated by the probable presence in the half spindle of two materials, spindle fibers and interfibrillar substance. If there are two such materials present, the mitochondria or other cytoplasmic granules that make their way into the spindle by brownian movement would be expected to occupy the region of lower viscosity, i.e., the interfibrillar region. Under such conditions their speed of movement would be determined by two factors: the viscosity of the interfibrillar substance and the amount of space available for movement between the fibers, especially if this were very limited.

Bělař (1929b) found that most spindles in cells of the stamen hairs of Tradescantia contained a few tiny granules in quite rapid brownian movement. This motion of the granules, which appeared first in the polar caps (clear regions that adjoin opposite sides of the nucleus at late prophase and in which the spindle later develops), was evident in the fully-formed spindle and continued undiminished up to the time of formation of the cell plate, when the granules disappeared from view. This contrasted with the situation he found in the spindles of animal cells, namely, nematode eggs, lepidopteran and grasshopper spermatocytes, and Actinophrys, in which granules of a comparable size in the spindle were always relatively quiet (see Bělař, 1929a). He interpreted this difference to indicate that in the Tradescantia cell there was a larger amount of the less viscous interfibrillar substance than in the animal cells he studied. I have not been able to confirm Bělař's observation that the granules show greater freedom of movement in the direction of the long axis of the spindle than at right angles to it, but this may mean only that the interfibrillar substance is more abundant and the fibers farther apart in my material than in his.

Ris (1943) reports unrestricted brownian movement of the cytoplasmic granules that make their way into the interzonal region during anaphase in embryonic cells of Tamalia. Chambers (1924) states that the spindle of the dividing sand-dollar egg has become "distinctly fluid" by the time the chromosomes have reached the poles. He doubtless refers to what I have termed the interzonal region. These observations agree well with the conditions in the grasshopper neuroblast.

Possible causal factors in viscosity change

With regard to the factors responsible for viscosity changes of the cytoplasm, two possibilities seem deserving of consideration: alterations in the water content and in the pentose nucleic (ribonucleic) acid content of the cytoplasm.

It has already been pointed out (p. 115) that the first detectable viscosity drop coincides with the initiation of the "rounding-up" of the neuroblast in late prophase and that the viscosity is rising as the cell flattens on one side to assume the hemispherical form during telophase. Since it can be demonstrated that immersion of these cells in hypotonic culture medium causes a fall in viscosity owing to intake of water and that hypertonic medium causes a rise in viscosity through loss of water, it seems not unlikely that the late prophase viscosity fall and "rounding-up" of the cell might result from the intake of water, while the telophase viscosity rise and the accompanying flattening of the cell at one side might be due to loss of water. Unfortunately, the irregularities in the hemispherical-shaped cell make it impossible to determine whether there is any actual change in cell volume during the alteration in shape. Chambers (1919) relates the change in form of the Arbacia egg from spherical to hemispherical following the first cleavage division to a viscosity shift, but he associates it with a lowering of the viscosity.

Water exchange seems inadequate, however, as a complete explanation of the observed viscosity changes for two reasons. First, it seems doubtful that the intake of water would occur in sufficient amounts to account entirely for the observed viscosity fall. Second, the viscosity continues to rise for some time after the cell has returned to its hemispherical shape, when the water loss might be supposed to have been completed. Another factor, therefore, would appear to be involved, either in place of or in addition to water intake and loss.

In support of the possibility that changes in the pentosenucleic acid content of the cytoplasm may be at least partly responsible for the observed viscosity changes is the fact that pentosenucleic acid, which is known to be present in large quantities in the cytoplasm of rapidly growing tissues (Brachet, 1933; Caspersson and Schultz, 1940) and which has a strikingly high viscosity ($\eta = 62.4$, according to Cohen and Stanley, 1942), undergoes a change in amount per cell during mitosis (Brachet, 1940; Caspersson, 1940; Painter, 1943). Both Brachet, from studies of the eggs and early developmental stages of different animals, and Painter, using Rheo meiotic cells, reached the conclusion that cytoplasmic pentosenucleic acid is abundant in mitotically active cells at early prophase, less abundant or entirely absent from late prophase through anaphase, and increasing in amount following division. They believe that the late prophase decrease is due to transformation of pentosenucleic acid into the desoxypentosenucleic acid of the developing chromosomes and that the increase following division is due to the loss of desoxypentosenucleic acid from the chromosomes and its transformation into pentosenucleic acid. These changes in the nucleic acid content of the cytoplasm during mitosis bear a very close resemblance to my viscosity curve for the neuroblast cytoplasm. If there is any discrepancy, it would seem to be the exact time during late prophase that the nucleic acid leaves the cytoplasm in detectable amounts, and neither the studies of Brachet nor Painter furnish information on this point. The statement of White (1942), however, that "the nucleic acid of the chromosomes undergoes a sudden increase at prometaphase, when the nuclear membrane breaks down and substances from the cytoplasm have free access to the chromosomes" suggests that the fall in cytoplasmic nucleic acids may coincide closely with the viscosity fall of the cytoplasm. The viscosity rise, which extends through all of telophase, could be accounted for, up to the time of nuclear membrane formation, by a return of the pentosenucleic acids to the cytoplasm during the retrogressive chromosome changes, and, after re-constitution of the nuclear membrane, by the synthesis of new pentosenucleic acid.

SUMMARY

Observations on the rapidity of brownian movement of the mitochondria in different regions of the grasshopper neuroblast during the entire mitotic cycle indicate that:

1) The viscosity of all parts of the cytosome is relatively high during interphase and prophase, begins to fall in late prophase, reaches a minimum at anaphase, and rises gradually to its original high level during telophase.

2) The viscosity of the portion of the spindle between the pole and the plane in which the proximal ends of the chromosomes are situated appears to fall slightly from a high prometaphase level through anaphase.

3) The viscosity of the portion of the spindle situated between the separating daughter chromosome groups shows, during anaphase, a slight drop from a medium value, and this is followed at the beginning of telophase by an abrupt rise to a very high level, which is maintained through early and middle telophase.

Alterations in water content and nucleic acid content of the cytoplasm are suggested as possible explanations of viscosity changes during mitosis.

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TOXIC EFFECTS OF COPPER ON ATTACHMENT AND GROWTH OF BUGULA NERITINA[1]

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INTRODUCTION

This paper describes physiological experiments dealing with the effect of copper on attachment and early development of *Bugula neritina* (L.), a widely distributed marine fouling organism. The studies were designed to gain a clearer understanding of the specific role of copper in prevention of fouling and the mechanism of action of copper paint surfaces. Various experiments using copper solutions and paints were made to gain an integrated concept of copper toxicity.

Copper, in one form or another, has been used since ancient times to prevent fouling and is one of the most effective metallic poisons for marine fouling organisms (Visscher, 1927). The antifouling efficiency of metallic poisons is related to the intrinsic toxicity of their ions and the solubility of their corrosion products in sea water, both relatively high for copper (Parker, 1924). Recently, this concept has been clarified and extended by Ketchum, Ferry, Redfield, and Burns (1945) who show that the prevention of fouling is related to the concentration of toxic dissolved in the water at the surface of the paint, and that the rate of loss of toxic is a measure of the steady-state concentration in a narrow zone at the paint surface. The rate of loss of toxic from a paint surface is known as the leaching rate. The authors show that in the case of copper paints, a leaching rate maintained consistently above a minimum of 10 micrograms of copper per square centimeter per day will prevent serious fouling.

Much discussion and some experimentation has centered around the question of the relative toxicities of different forms of copper. Carritt and Riley (1943), however, were unable to demonstrate any difference in toxicity to *Bugula turrita* between cuprous and cupric ions, while Clarke (1943) found that "all forms of copper have roughly the same toxicity but differ greatly in their solubilities."

The establishment of fouling organisms on a submerged surface depends *a priori* on their ability to affix themselves and to develop after attachment. Effective antifouling surfaces, therefore, must function in one or both of two ways, by repelling (or killing) larvae of fouling organisms, or by inhibiting development of any that attach. Visscher (1927), Neu (1932), and Edmondson and Ingram (1939) conclude from indirect evidence obtained mainly in field studies that toxic paints have no effect on growth of fouling organisms once attached, that they function only by repelling or delaying attachment. Without minimizing the repellent action of antifouling paints, evidence is accumulating to show that toxic surfaces function importantly through inhibition of growth of attached larvae. The ingenious experiments of Pomerat and Weiss (1943) showing inhibition of fouling growth on unpainted areas by adjacent toxic paint surfaces strongly support this view. Similar "distance effects" of antifouling paint surfaces on fouling growth on adjacent untreated areas are frequently seen in panel tests (e.g., Edmondson, 1944, Fig. 7a, p. 13).

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[1] These studies were conducted at the Naval Biological Laboratory at San Diego, California, under direction of Mr. W. F. Whedon to whom acknowledgment is due for many suggestions. The author is indebted to Dr. Alfred C. Redfield of the Woods Hole Oceanographic Institution for helpful advice and criticism. Acknowledgment is also made to Mr. C. J. Rapean and Miss Mildred Marshall for making the chemical analyses, and to Dr. Easter C. Cupp for valuable assistance. The Navy Department has given permission to publish the results. The opinions contained herein do not necessarily reflect the official opinion of the Navy Department or the naval service at large.

Toxic effects of copper on various fouling organisms have been described by Bray (1924), Whedon *et al.* (1942 and 1943), Miller and Cupp (1942), Clarke (1943), and Riley (1943) in unpublished reports to the Bureau of Ships, U. S. Navy Department. Prytherch (1934) reports stimulating effects of small amounts of copper on settling and metamorphosis of the oyster, and similar oligodynamic effects on ascidians were observed by Grave and Nicholl (1939).

Jones (1941) reviews theories on the mechanism of toxic action of metals, notably copper. These may be divided into two main groups which maintain that: (1) copper retards vital processes through inactivation of essential enzymes, and (2) copper acts more directly by precipitating cytoplasmic proteins as copper-proteinates. With regard to fouling organisms, Clarke's (1943) studies on barnacles and mussels, and Riley's (1943) investigations and those of the present author on bryozoans favor the inactivation theory. Further studies are needed, however, to elucidate the physiological mechanisms involved.

The results of the present investigation will be presented in four parts: (1) attachment and growth of *Bugula* on copper paint surfaces, (2) growth of *Bugula* in copper solutions, (3) recovery of *Bugula* from copper poisoning, and (4) toxicity gradients of copper paint surfaces.

Materials and Methods

Bugula larvae for these studies were obtained as follows. Mature colonies were collected from docks, piling, etc., in San Diego Bay the day before tests were made, and placed in aquaria of sea water in the laboratory. Early the next morning, these liberated swarms of larvae which were easily collected at the light side of the container since they are positively phototropic and visible to the naked eye (about 0.2 mm. in length and darkly colored). *Bugula* larvae normally attach and begin to grow before mid-afternoon of the day on which they are liberated as described by Grave (1930) and Edmondson (1944). In subtropical localities, as San Diego Bay, larvae may be obtained the year around. From the foregoing facts, it is obvious that this form is remarkably suited for studies on fouling problems.

Primed steel, 1 × 3 inch panels were coated with the various experimental and control paints employed in the first section of this paper. Larger panels (10 × 12 inches) were similarly prepared and exposed in the bay to test their antifouling efficiency under field conditions. In most cases, the small panels were "seasoned" before testing by soaking them for a month or more in large jars of sea water. The seasoning bath was changed at frequent intervals and aerated by means of a stream of air bubbles delivered at the base of the container by means of a tapered glass tube.

For other studies, larvae were allowed to attach to small test panels which were coated with a non-toxic paint composed of equal parts of paraffin and ester gum. So attached, they could conveniently be transferred to the experimental situations or be removed therefrom for observations.

Other procedures will be described as necessary in the various sections of the investigation.

Attachment and growth of Bugula on copper paint surfaces

The first problem was to determine the effect of actual copper paint surfaces on attachment and growth of *Bugula* larvae under simulated natural conditions. The data given are typical of many such tests that have been performed.

TABLE I

Attachment and growth of Bugula larvae on hot and cold plastic paints, each series with graded copper content. (Paints seasoned for two months in sea water bath before tests were made.)

Series	Paint No.	Percentage Cu_2O in dry paint	Leaching rate (2-mo.) μg Cu/cm.^{2}/day		Per cent larval attachment ± S.E.		Growth (3-day) Maximum length[1] (mm.)	
			Filmed	Clean	Filmed	Clean	Filmed	Clean
Hot plastic	1	37.0	28.1	24.1	4±1.6	2±1.1	0.3	—
	2	32.0	19.4	23.7	23±5.3	15±4.4	0.3	0.3
	3	26.0	14.0	14.0	5±3.3	13±6.3	0.2	0.2
	4	19.0	13.5	11.5	79±3.5	72±5.0	0.2	0.2
	5	10.6	6.9	5.8	86±4.4	95±2.6	0.4	0.6
	6	0	—	—	100	100	1.3	1.2
Cold plastic	7	36.0	14.3	14.1	0	3±2.4	—	0.2
	8	30.0	14.1	13.3	5±3.0	1±1.4	0.2	0.2
	9	19.2	14.1	13.0	7±2.4	10±4.5	0.3	0.2
	10	9.3	3.5	3.8	87±3.6	73±6.8	0.6	0.7
	11	0	—	—	78±7.4	98±1.4	1.1	1.1

[1] As attached larvae are approximately 0.2 mm. in length, maximum growth increments may be calculated by subtracting this value from the maximum lengths given above.

Two series of copper paints (one hot plastic and one cold plastic series)[2] were prepared, each having the same matrix composition but graded amounts of cuprous oxide (Table I). Two test panels were coated with each of these paints and seasoned for ten weeks. Prior to testing, one panel in each duplicate set was wiped to remove the slime film that accumulated during seasoning; the other was used without being cleaned.

At the start of the attachment test, the panels were immersed in jars containing 700 cc. fresh sea water and immediately thereafter a known number of *Bugula* larvae (usually 100) were added. Tests were always started in mid-morning when the larvae were active, and counts of the number attached were made late in the afternoon when all larvae were either attached or dead. The percentage of attachment was calculated by dividing the number of larvae affixed to the test panel by the total number of larvae used.

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[2] A hot plastic paint is one that is applied hot and forms a solid film on cooling; cold plastic paints are one of the types of paints which dry by evaporation of the solvent. The paints used in these experiments were experimental modifications of standard Navy formulations.

Data on development, which involves both growth and differentiation, were obtained by measuring the length from base to apex of the young *Bugula* stalks, and by determining the number of polypides developed from time to time. Length measurements were made with an ocular micrometer under low magnification.

The results of the attachment and growth tests on the various copper paint surfaces are given in Table I and in Figures 1 and 2. The data clearly segregate two groups of paints. Those with less than 15 per cent Cu_2O content or leaching rates less than 10 micrograms of copper per square centimeter per day are obviously inferior as over 70 per cent of the larvae were able to attach to them *and* to grow significantly. These paints foul in a more or less short period of time in field tests. The second group, with greater copper content and concomitantly higher leaching rates, were significantly more efficient in repelling attachment and permitted no significant growth. These paints are more effective in the field, the length of their effective periods being related to their copper content.

That leaching rate rather than copper content is responsible for the antifouling performance of a paint can easily be demonstrated by comparing *Bugula* attachment and growth on surfaces with identical amounts of cuprous oxide but different leaching rates. If, for example, ester gum is substituted on an equal weight basis for rosin as the resinous component in our hot plastic series, leaching rates fall far below the adequate level and *Bugula* larvae attach abundantly and develop colonies on such surfaces. Ketchum *et al.* (1945) have shown that substitution of ester gum, esterified Albertol or coumarone-indene for the rosin content of an effective antifouling paint (Navy Department Specification 52-P-161) drastically reduces the leaching rate below the critical level with the result that such paints foul quickly in the field. Thus, copper content is important only insofar as it contributes to an adequate and sustained leaching rate, and may be neglected in the subsequent discussion.

In these tests, the slime film on the various paint surfaces had no consistent effect on larval attachment. Its presence apparently is not necessary for attachment of *Bugula* since larvae affixed themselves to cleaned panels as well as those with a coating of slime film, and since they readily attach themselves to unseasoned surfaces shortly after their initial submergence and certainly before any visible film forms. Whether or not an invisible film is prerequisite to attachment is an academic question. In the tests cited, the larvae had no choice between surfaces with and those without slime film. When such choice is involved, *Bugula* larvae do exhibit certain preferences (Whedon *et al.*, 1943). The role of the slime film on attachment to toxic and non-toxic surfaces under various experimental conditions will be considered in another paper.

Larval attachment to paints with leaching rates above 15 micrograms of copper per sq. cm. per day ranged between zero and 23 per cent with an average of less than 10 per cent. None of these was able to grow, however, and hence to establish a colony. The range of leaching rates between 10 and 15 μg. per sq. cm. per day may be regarded as a "marginal zone," especially as the lower value is approached, since paints with leaching rates in this range often permit considerable attachment of larvae. These may or may not be killed. If not, as will be shown later, some of them might recover and grow should the leaching rate and subsequent surface concentration of toxic fall below the adequate growth inhibiting level. Even if the attached larvae are killed, however, there is still the possibility that their dead bodies might form a less toxic substrate for later larvae than the paint surface itself. Larvae frequently attach themselves on top of other larvae, often in clusters. Conceivably, those underneath might take up the toxic ions diffusing from the paint or in other ways shield the overlying larvae so that the latter could develop. Certainly, those on top would be further removed from the paint surface and hence would be located in a less concentrated part of the toxic zone which might permit

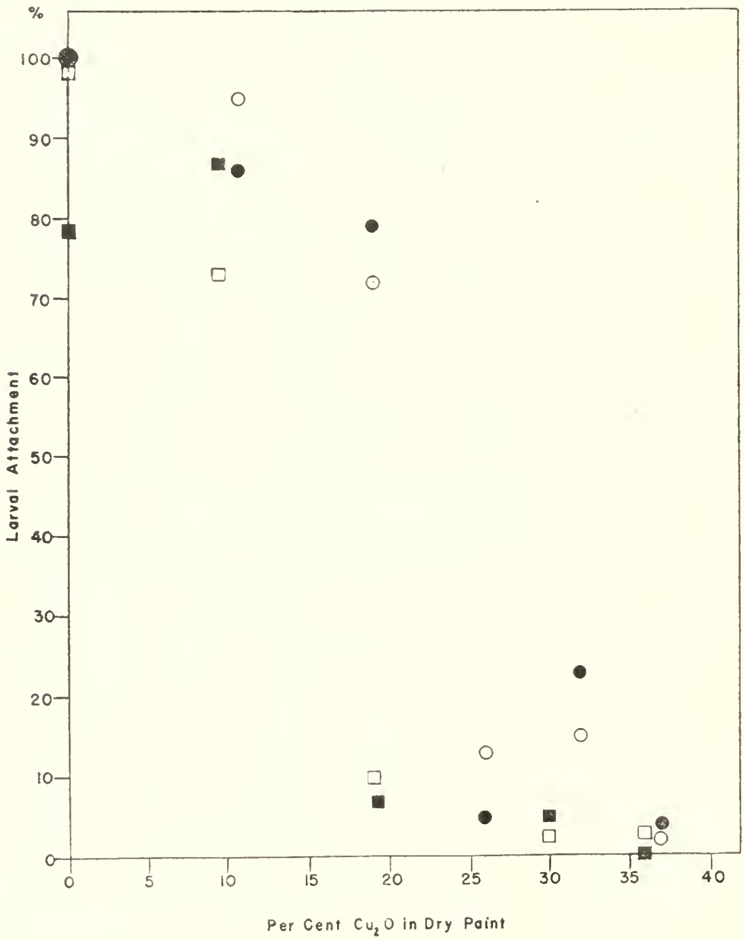

FIGURE 1. Attachment of *Bugula* larvae in relation to copper content of paint. Circles represent hot plastic paints while squares indicate cold plastic paints. Black-filled data points represent paint surfaces coated with slime film while clear data points represent cleaned surfaces (wiped before testing). All paints were seasoned for ten weeks in sea water before testing.

their development. While this sort of attachment is obviously not very secure, firmer attachment may eventuate by means of stolonic outgrowths from developing colonies to the paint surface. Such root-like processes are developed by erect bryozoans and may give rise to secondary colonies.

The fact that at least a small percentage of larvae attach at any one time to quite toxic surfaces indicates that under field conditions the growth inhibiting function of toxic surfaces plays an important role in the prevention of fouling. Effective antifouling surfaces must function in part by stopping development of attached larvae. This is contrary to the previously mentioned views of Visscher, Neu, and Edmondson and Ingram. The latter authors report a restricted growth of *Bugula* and serpulid worms on toxic surfaces exposed in Hawaiian waters, but attribute this to "delayed attachment rather than to a slower rate of growth after settling."

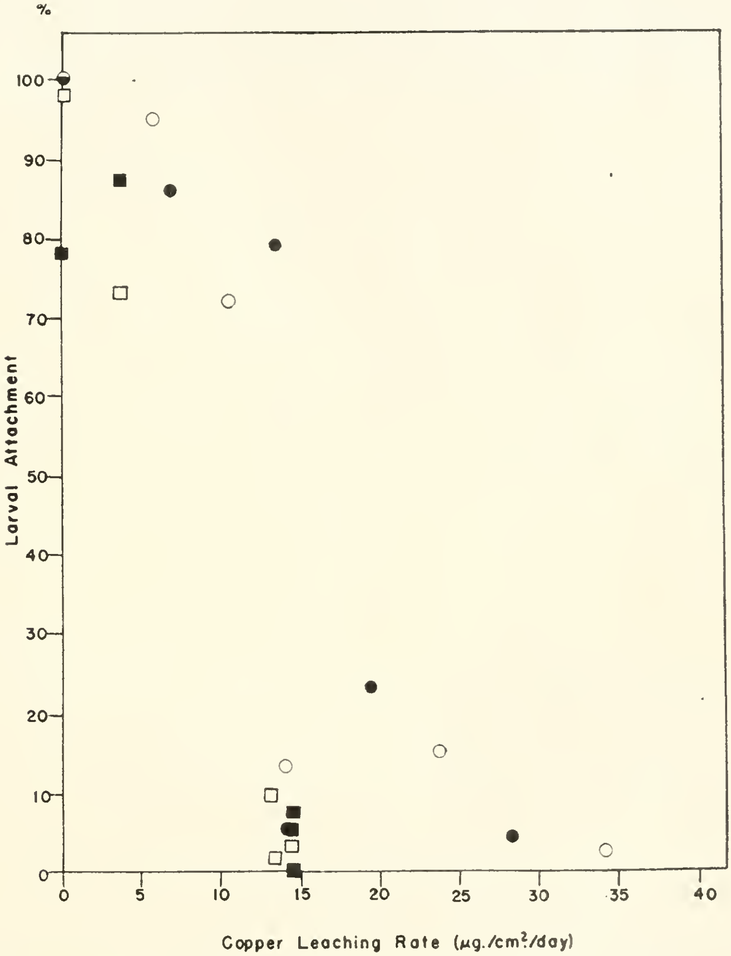

FIGURE 2. Attachment of *Bugula* larvae in relation to copper leaching rate. Symbols as in Figure 1.

Dates of attachment in their tests are not known, and hence rates of growth on treated and untreated surfaces cannot be calculated and compared from their data. In view of the present studies, it would seem that the alternate interpretation of a growth retarding effect is equally plausible, if not more probable.

To illustrate further the growth retarding effect of toxic surfaces, another experiment involving growth of *Bugula* on unseasoned copper paint surfaces may be cited. In this experiment, the first two weeks of growth of *Bugula* on two hot-plastic, copper-paint surfaces (*A* and *B*) was compared to that on a non-toxic, hot-plastic control (*C*). The two toxic paints have identical copper content (32 per cent Cu_2O), but paint *A* (w.w. rosin-paraffin matrix) has an excellent field record correlated with adequate leaching rates, while paint *B* (ester gum-paraffin matrix) fouls quickly in the field because of low leaching rate. Hot-plastic paints of the type here used have low initial leaching rates and do not develop characteristic leaching rates until they have been submerged for a period of time. Hence, *Bugula* larvae attach abundantly to unseasoned panels coated with these paints and begin to grow normally on them.

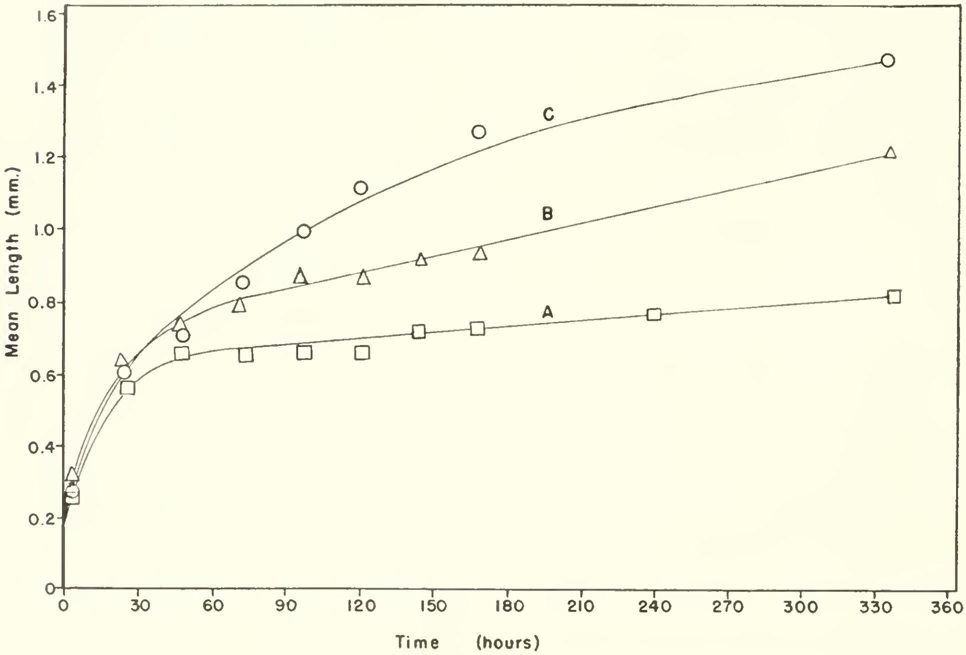

FIGURE 3. Growth of *Bugula* ancestrulae on unseasoned hot-plastic paints. *A* is an excellent copper paint with adequate leaching rate, *B* is an inferior paint with low copper leaching rate, and *C* is a non-toxic control.

As shown in Figure 3, the growth curves in the present experiment do not begin to diverge significantly until after the second day. Growth on paint surface *A* was practically stopped at this time at the first polypide stage. Some individuals developed the first polypide, most did not, and no colonies were established. Colonies were developed on paint surface *B*, but growth and differentiation were significantly less than for controls. The three curves become more and more divergent with time.

The foregoing differences between growth inhibiting properties of paints *A* and *B* must be attributed to differences in their leaching rates as their copper content and growth of *Bugula* on their respective controls are identical. Repeated leaching rate determinations of these paints have shown both to have low initial leaching rates (which accounts for the initial similarities in the *Bugula* growth curves for the two surfaces) but paint *A* eventually develops an adequate leaching rate, while leaching rates of paint *B* do not attain the adequate level. Thus, again the growth retarding property of copper paint surfaces is demonstrated and associated with the copper leaching rate.

TABLE II

Effect of copper on growth and development of Bugula ancestrulae

Av. copper concentration mg./liter	Growth and differentiation in copper solutions				
	Mean length increments ± S.E.			Polypides per colony	
	1 day	3 days	5 days	3 days	5 days
	mm.	mm.	mm.		
Controls	0.36±.007	0.61±.016	0.77±.021	1–2	1–2
0–.05	0.30±.015	0.55±.013	0.63±.018	1	1–2
0.05–.10	0.26±.009	0.37±.010	0.46±.012	1	1–2
0.10–.15	0.25±.007	0.36±.008	0.48±.013	1	1
0.15–.20	0.14±.008	0.22±.009	0.24±.011	0–1	0–1
0.20–.25	0.08±.007	0.10±.008	0.12±.010	0	0
0.25–.30	0.02±.007	0.03±.008	0.02±.009	0	0
0.30–.35	0.04±.011	0.03±.011	0.03±.009	0	0
0.35–.40	–0.03±.008	0.01±.009	0±.008	0	0
0.40–.45	0.03±.007	0.02±.011	–0.03±.010	0	0
0.45–.50	0.02±.008	0.03±.010	0.03±.010	0	0

The experiments cited in this section are in close accord with results obtained by Ketchum *et al.* (1945) and substantiate the leaching rate theory of action of antifouling paints. It is noteworthy that their estimate of minimum adequate copper leaching rate (10 micrograms per sq. cm. per day), which was based on comparisons of numerous leaching rate determinations with field exposure tests, is confirmed by the *Bugula* attachment and growth tests. Data presented in this section show that copper paints with leaching rates less than this value permit larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 10 micrograms per sq.cm. per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms of copper per sq. cm. per day, but these do not develop into colonies.

Growth of Bugula in copper solutions

To determine more precisely the effect of copper ions on growth of *Bugula,* nontoxic panels bearing attached larvae (about 25) were immersed in sea-water solutions of graded copper concentrations. Cuprous oxide was the salt used in making the copper solutions, but the ions were presumably cupric since the oxidation of cuprous ion is supposedly rapid. The young *Bugulae* were measured just before immersion in the experimental solutions and at one, three, and five day intervals thereafter. Polypide development was also observed at these times. Mean length increments after immersion were computed as an index of toxicity of the solutions.

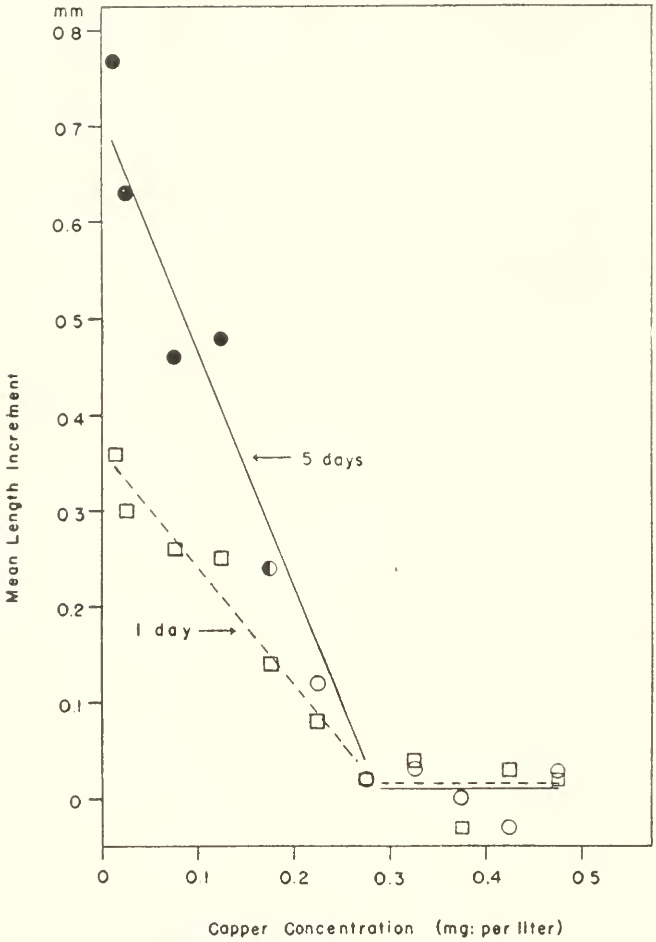

FIGURE 4. Growth of *Bugula* ancestrulae in relation to copper concentration. Curves for one-day (squares) and five-day increments (circles) based on grouped data (class intervals = 0.05 mg. copper per liter). Filled squares indicate one or more polypides per colony, half filled squares show that some stalks did not differentiate polypides, and open data points indicate that no polypides were developed. See also Figure 5.

The copper concentrations of each solution were determined before and after the experiment, and the average values were used.

As clearly shown in Table II and Figures 4 and 5, the degree of early growth and differentiation of *Bugula* is closely related to the copper concentration in the water surrounding the organisms. Up to about 0.3 mg. per liter, increment in length is inversely proportional to copper concentration of the solution. Higher concentrations inhibit growth completely or allow but slight, insignificant increment. The sharp break or inflection in the curves in Figures 4 and 5 at about 0.3 mg. copper per liter apparently marks this as a critical concentration for growth. No stimulating effect of small amounts of copper on growth was noted, since even in the greatest dilutions used growth increments were less (with minor exceptions) than in fresh sea-water controls.

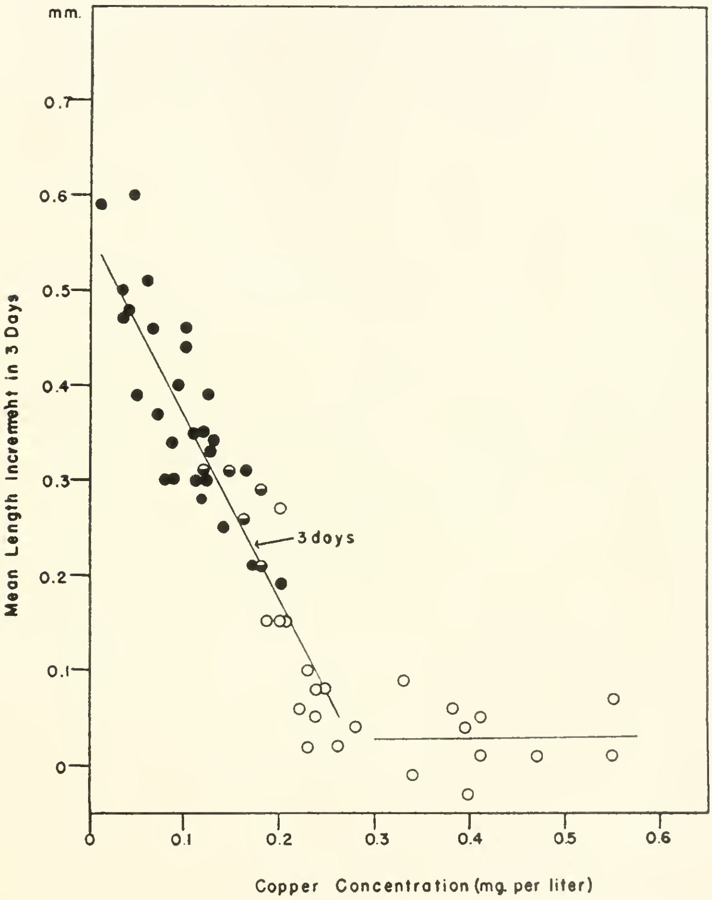

FIGURE 5. Three day growth of *Bugula* ancestrulae in relation to copper concentration. Black circles indicate fully developed polypides, half filled data points represent partly formed polypides, and clear circles indicate no differentiation of polypides. See also Figure 4.

Polypide differentiation is inhibited by copper concentrations greater than 0.2 mg. per liter, the minimal value being less than that required to prevent growth completely. Concentrations less than 0.2 mg. per liter only delay polypide development. This is shown by the fact that only controls attained the second polypide stage at three days (Table II), by the several cases of incomplete or nonfunctional polypides at three and five days in the range between 0.1 and 0.2 mg.

per liter (Figs. 4 and 5), and by the fact that at five days only the first polypide was developed in concentrations greater than 0.1 mg. per liter.

A series of critical copper concentrations may be postulated from these and other studies (Table III). The minimum lethal dose (MLD) for free swimming larvae is about 0.3 mg. per liter (Miller and Cupp, 1942), the same concentration required to stop growth of attached larvae. No data are available on concentrations necessary to prevent larval attachment. Presumably these would be at least as great as the minimum lethal dose, and probably greater, since larvae in solutions containing as much as 0.4 to 0.5 mg. copper per liter do not die immediately but swim about more and more slowly for a half hour to an hour or more. Given a suitable surface, it is conceivable that some might attach in this interval.

TABLE III

Critical copper concentrations

Copper concentration (mg. per liter)	Physiological effect
>0.3	Kills larvae. Inhibits growth.
0.2–0.3	Retards growth. Inhibits polypide development.
<0.2	Retards growth and development of polypides.

Recovery of Bugula from copper poisoning

The question arises, are the above-described toxic effects of copper permanent, or can organisms recover to any significant degree after exposures to sublethal dosages? Preliminary experiments showed that *Bugula* ancestrulae can recover nearly normal development after immersion for 6 to 24 hours in sublethal copper sea-water solutions (Miller and Cupp, 1942). In this experiment, the periods of immersion in copper solutions were extended (three to seven days) and alternated with periods of immersion in fresh sea water to determine the effect on recovery of longer exposures and of intermittent as compared to continuous dosages.

The initial procedure was similar to that of preceding experiments. A non-toxic test panel with attached larvae was immersed in each of eight copper sea-water solutions (Nos. 1–8, Table IV) for a period of three days (time period I). The subsequent procedure features a staggered schedule of transfers of the animals from copper solutions to fresh sea water, so that, while one group was exposed to copper, the other was immersed in untreated sea water and *vice versa*. After the first three days exposure, half of the panels (group *A*, sets 1–4) were transferred to fresh sea water, and the remainder (group *B*, sets 5–8) were left in the original copper solutions. After four days (period II), group *A* panels were transferred from sea water back to the original copper solutions, while group *B* sets (that had now been in the toxic solutions for a week) were transferred to fresh sea water. After another four days (period III), the process was again reversed—those in sea water were transferred back to copper solutions and *vice versa*. The experiment was terminated on the fifteenth day, the final four days constituting period IV.

Length measurements were made before the original immersion (a few hours after attachment of the larvae), at the time of transfers (on the 3d, 7th, and 11th days), and at the termination of the test on the 15th day. From these data, the rates of growth relative to that of controls for the four time periods in copper solutions and sea water were computed. Concentrations of the copper solutions were estimated from their biological effects (using curves in Figs. 4 and 5) as chemical determinations were not made for this test. Data are given in Table IV, and the salient features are shown in Figure 6.

In all but one case, transfers to fresh sea water were followed by marked and significant increases in growth rate and polypide differentiation, while the reciprocal transfer resulted in sharply decreased, if not completely inhibited, growth (Fig. 6). The exceptional case (set 7) presumably received a lethal dose in the first period as no significant increases in length and certainly no differentiation were observed following transfer to fresh water. All others, however, exhibited an amazingly high degree of plasticity and regulatory ability in recovering from the effects of the poison. Even those immersed an entire week in solutions that allowed but slight growth increment and no visible differentiation (e.g., sets 5 and 6) were able to recover significantly, from growth rates of zero to as much as 60 per cent that of controls, when restored in fresh sea water. They also differentiated polypides though these were slightly abnormal in some instances. Set 8 in group *B* was apparently exposed to a weaker solution than any of the others since these individuals not only grew considerably but also developed polypides even while immersed in the copper solution. Nevertheless, significant increases and decreases in relative growth rates for this set were observed in the various periods. Seemingly, one could control rate of growth and differentiation almost at will by appropriate exposures to copper. The ability of young *Bugulae* to recover each time after repeated and long immersions in rather toxic copper solutions is truly remarkable.

Table IV

Growth and development of Bugula ancestrulae during and after immersions in copper sea-water solutions

Symbol No.	Cu. in solns. mg./liter	Mean length increments, time periods I–IV *				Total no. of polypides per colony at end of periods:			
		I (3 days) mm.	II (3–7 days) mm.	III (7–11 days) mm.	IV (11–15 days) mm.	I	II	III	IV
A-1	0.26	0.05	0.34	0	0.14	0	1	1	2
-2	0.27	0.04	0.22	0.01	0.09	0	1	1	2
-3	0.24	0.09	0.27	0.10	0.14	0	1	1	2
-4	0.23	0.11	0.25	0.09	0.06	0	1	1	2
B-5	0.25	0.08	0.01	0.12	−0.02	0	0	1 ab.	1
-6	0.23	0.10	0.01	0.17	−0.02	0	0	1 ab.	1
-7	0.3	0.03	0.03	0.05	0	0	0	0	0
-8	0.20(?)	0.14	0.16	0.23	0.10	1	1	1	2
Control		0.68 (0.68)	0.64 (0.53)	0.11 (0.28)	0.25 (0.19)	1	2	3	4

* Group *A* immersed in copper solutions during periods I and III, and in fresh sea water during periods II and IV. Group *B* immersed in copper solutions during periods, I, II, and IV, and in fresh water during period III.

In no case was recovery complete as judged either by growth rates or polypide development. Growth rates after transfers to sea water ranged roughly between a third and four-fifths of the normal (see Fig. 6), and the number of polypides developed was less than that of controls.

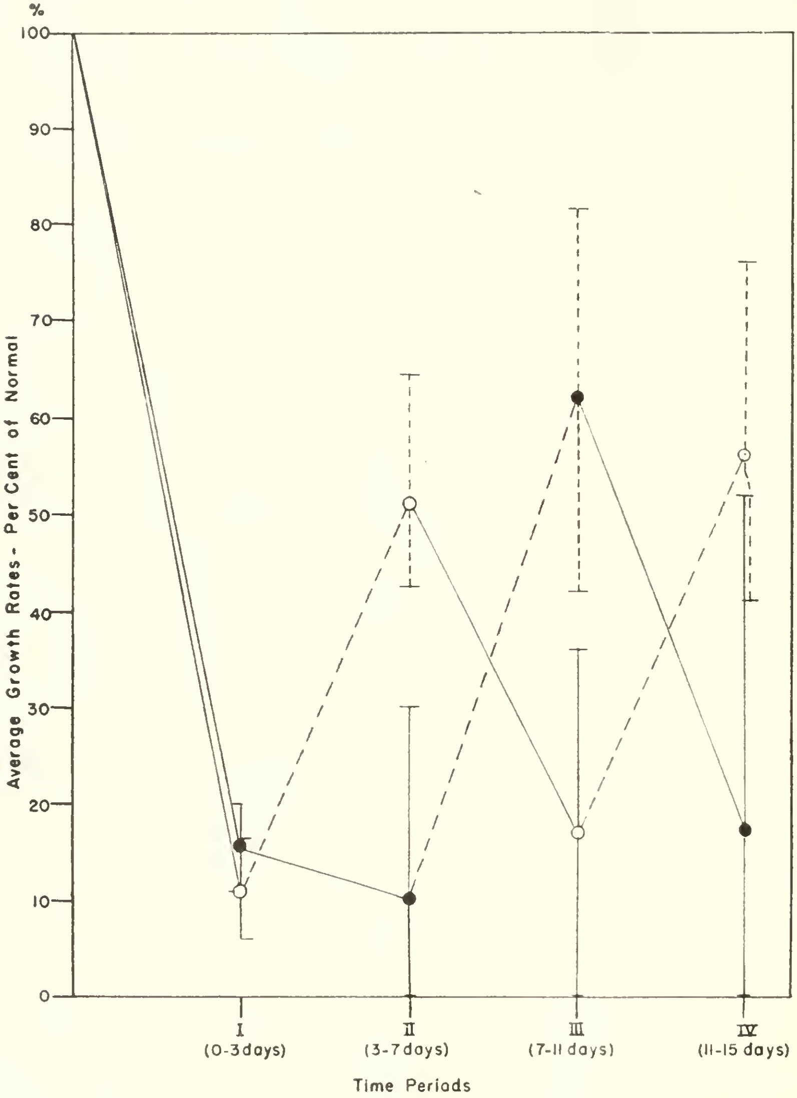

FIGURE 6. Growth rates of *Bugula* ancestrulae (in per cent of normal) during and after exposure to sublethal copper concentrations. Black data points represent averages for group *A* panels (Table IV), and clear circles are averages for group *B* panels (omitting set 7). Solid lines indicate periods of immersion in copper solutions, and dashed lines represent transfer to fresh sea water. Vertical lines through data points show the range between minimum and maximum growth rates for each average.

Length of exposure in sublethal concentrations has relatively little apparent effect on recovery. This is shown by the fact that *Bugulae* exposed to copper for seven days were able to develop after transfer to sea water at a rate comparable to that of specimens which had been exposed for only three days. As previously noted, however, the longer exposures caused abnormal polypide development in some cases.

In contrast to this finding, there is evidence that in the case of barnacles and mussels the length of exposure is as important as the concentration in determining the toxic effects of copper solutions. Clarke (1943) found that low concentrations acting for a long time produced effects equivalent to high concentration acting for a short time. In other words, the toxic effect is proportional to the product of duration and intensity. Further experiments of this general type are needed to determine more precisely immediate and long-term effects of various exposures and concentrations and of continuous versus discontinuous exposures.

These experiments have practical implications since, under natural conditions, the concentration of toxic ions adjacent to a copper paint surface might vary from time to time as a result of fluctuation in leaching rate, formation of surface films, currents flowing past the surface, and other factors. The effect of such changes on attached *Bugula* larvae might be surmised from the foregoing studies showing that growth is an inverse function of copper concentration in the medium and that stunted individuals can resume development when restored to non-toxic situations. Presumably, *Bugula* ancestrulae attached to a surface with fluctuating toxicity might recover to some degree with each significant decrease, if the organisms were still viable. With each increase in length, the ancestrulae greatly improve their chances to establish colonies for reasons which will become apparent in the next section of this paper.

Toxicity gradients of copper paint surfaces

The question of zones or gradients of toxicity adjoining toxic paint surfaces is involved in understanding their antifouling action, and is the last problem to be considered in this paper. Although a toxicity gradient has been assumed as a consequence of diffusion of toxic ions emanating from an antifouling paint, a demonstration of this seemed desirable. Furthermore, information on the effective limits and other characteristics of the toxic zone is of particular interest in connection with the establishment of colonial fouling organisms (e.g., erect bryozoans and most hydroids) that grow, plant-like, more or less perpendicularly away from the surface to which they attach, and develop new individuals at the ends of their branches. It would be useful to know how far from the toxic surface growth inhibiting and growth retarding concentrations are maintained, or how much a colony would have to grow before its terminal polypides were out of danger from poisoning. In the following experiment, the problem was to demonstrate, if possible, the existence of the toxic zone of an antifouling paint and to determine its general characteristics.

The preceding studies, showing rather precise relationships between growth of *Bugula* ancestrulae and copper concentration, suggested a method for attacking the problem of toxic gradients. The essential features of the procedure used and of the results obtained are illustrated in Figure 7. A non-toxic panel bearing attached and growing *Bugulae* was placed perpendicularly against a panel coated with a cold-plastic copper-paint (Table I, No. 7). With this arrangement, the developing *Bugula* stalks maintain practically constant distances between their axes and the toxic surface since they grow parallel to the latter. The non-toxic panel was ruled in millimeter divisions paralleling the toxic surface, and these were used as class intervals of distance in analyzing the data. The ancestrulae in each division were measured at the start of the tests and two days afterwards to determine the effect of diffusing copper ions on their growth at various distances from the paint surface. For control, a non-toxic panel was substituted for the toxic panel. Experimental and control racks were placed in an aquarium containing ten liters of sea water. The water was aerated during the tests by fine streams of air bubbles delivered through pinholes in a piece of rubber tubing stretched along the bottom of the aquarium. This method of aeration caused some circulation of water but no appreciable agitation.

TABLE V

Toxicity gradients of a copper paint demonstrated by growth of Bugula at measured distances from the toxic surface

Distance from paint surface	Test 1 Mean growth increment ± S.E. (2 days)		Test 2 Mean growth increment ± S.E. (2 days)	
	Experimental	Control	Experimental	Control
mm.	mm.	mm.	mm.	mm.
0–1	0.10±.02	0.48±.02	0.01±.01	0.43±.02
1–2	0.27±.03	0.42±.05	0.03±.01	0.46±.02
2–3	0.40±.06	—	0.16±.03	0.44±.03
3–4	0.38±.06	0.50	0.30±.06	0.49±.02
4–5	0.35±.10	0.50	0.40	0.50±.04
5–6	0.45±.08	0.45±.04	0.37±.04	0.50
6–7	0.55±.04	0.45±.04	0.35	—
7–8	0.45±.08	0.40	0.45	0.40
8–9	0.50±.07	0.50	0.35±.04	0.40
9–10	0.50	0.43±.03	0.41±.02	0.50

Two tests were made: the first, using toxic panels that had been seasoned for ten weeks in the laboratory; the second, with the same panels that were seasoned another two weeks. In the first tests, the slime film which had accumulated during seasoning was not removed but for the second tests the slime was wiped off. Data are given in Table V and graphically illustrated in Figures 7 and 8.

As clearly demonstrated by the retarded growth of the colonies near the painted surface, the toxicity arising from the surface decreases rapidly with distance from the surface. The outer effective limit of the toxic zone is variable. Beyond two millimeters from the surface in the first test and four millimeters in the second, no significant difference in growth between experimentals and controls was demonstrated. These values, then, represent the respective outer limits of the toxic gradient in the two tests and indicate the order of magnitude of the width of the toxic zone for a good antifouling paint. Within these zones, growth increments are roughly proportional to perpendicular distance from the toxic surface as might be expected from the diffusion gradient of toxic ions. Ancestrulae immediately

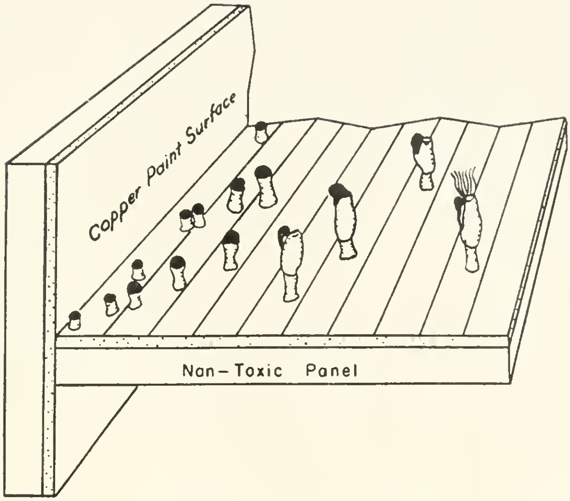

Figure 7. The toxic gradient extending from a copper paint surface as shown by growth of *Bugula* ancestrulae at measured distances from the toxic surface. *Bugula* figures are camera lucida drawings made four days after start of test 2 (for two-day growth, see Table V), and are twice enlarged in comparison to the millimeter rulings shown on the non-toxic panel.

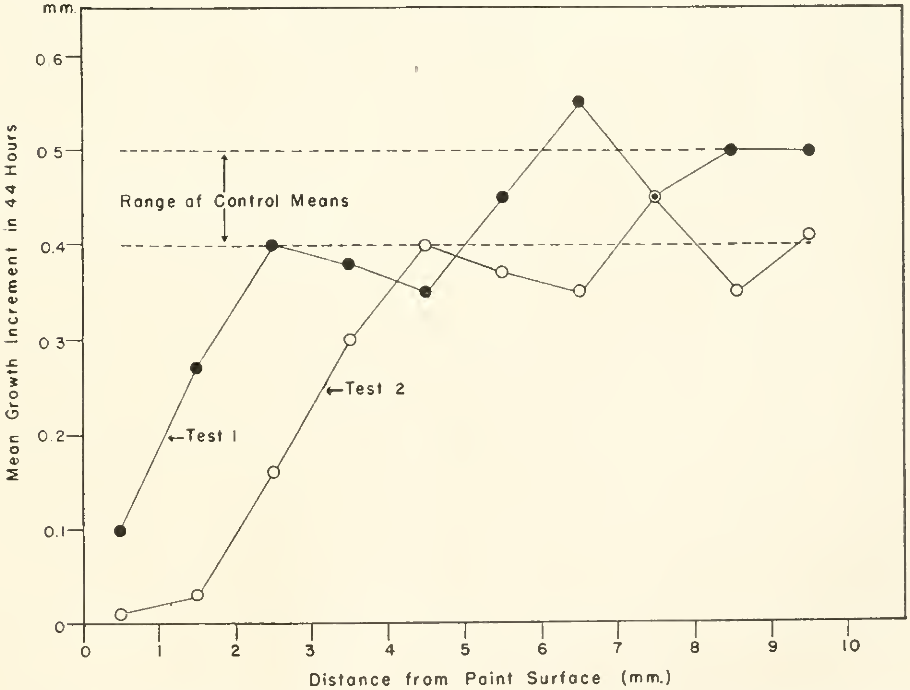

Figure 8. Gradients of toxicity of an antifouling paint: test 1—paint seasoned for ten weeks and coated with slime film, and test 2—after twelve weeks seasoning and with slime film removed before testing.

adjacent to the toxic paint, however, showed no increase in length indicating that growth inhibiting concentrations of copper were maintained there. This result was anticipated since larvae attached to surfaces coated with this paint do not grow (Table I, No. 7). Growth within the first millimeter interval is contributed by ancestrulae in the outer part of it. The width of the growth inhibiting portion of the toxic gradient (practically, the most important part of it) could be more precisely determined by further tests using smaller intervals of distance.

The panels used in the second test were clearly much more toxic than those first tested as shown by the greater width of the toxic zone, by the greater growth inhibition near the surface, and by the fact that the curve of growth increments at distances greater than four millimeters tends to fall below that of controls. With respect to the latter point, the mean growth increment for all individuals between four and ten millimeters in test two is significantly less than the corresponding mean in test one or that for controls. The means for single millimeter intervals in this range are not significantly different probably because of the small numbers of individuals in each.

The characteristics of the toxic zone are undoubtedly affected by various factors such as leaching rate and velocity of flow of water across the surface. In the field, currents or movement of the painted surface through the water would probably alter the character of the toxic zone. This and other factors could be simulated in the laboratory and their effects analyzed using the above-illustrated methods with appropriate modifications.

The demonstrated vertical gradient would probably differ from a horizontal gradient that extends outward from the edge of a painted surface. The latter type was nicely demonstrated by Pomerat and Weiss (1943) in field tests with panels on which areas of various shapes and sizes were left unpainted. The horizontal gradient observed by these authors was expressed both by graded growth of fouling on large unpainted areas, and by absence of fouling on smaller areas encircled by the paint. Their effects might be attributed in part to delayed larval attachment as well as retarded growth since the settling of larvae cannot be controlled in the field. For practical purposes, the activity of a vertical gradient is probably of greater importance.

The foregoing experiment together with those reported in preceding sections of this paper clearly indicates that the prevention of *Bugula* fouling on copper paint surfaces is dependent upon their ability to maintain growth inhibiting concentrations of copper in a narrow zone at the surface. The length of newly attached *Bugula neritina* larvae (about 0.2 mm.) presumably represents the minimum adequate width of the growth inhibiting zone required to prevent establishment of this organism. If attached forms are permitted to grow, their apical developing parts move away from the toxic surface and hence into regions of lower toxicity with consequent acceleration of development. As new polypides are produced by distal budding, they find themselves in a less toxic environment than their predecessors and eventually the terminal polypides would lie entirely outside of the toxic zone. Since the polypides are functionally independent (except for support), the colony can flourish even though its basal members are dead. If the toxic zone extends outward only a few millimeters, just a few of the basal polypides would be affected since *Bugula* colonies at the first polypide stage average about 0.9 mm. in length and each successive polypide adds at least a half millimeter to the length.

Larvae that settle on previously attached forms or on any inert particles elevated appreciably above the surface plane of the paint would clearly stand a better chance of survival than those attached directly to the toxic surface. They would occupy less toxic regions of the toxic gradient which might permit their growth, while those attached to the surface itself might be killed or permanently stunted by the higher concentration of the toxic prevailing there. Judging from the steep slopes of the toxicity gradients for an effective paint (Fig. 8), a fraction of a millimeter from the paint surface might make a significant difference in development, especially on surfaces with borderline toxicity.

To summarize: the foregoing preliminary tests clearly demonstrate a zone or gradient of toxicity that extends outward a few millimeters from an effective copper paint surface. Further experiments, using the method illustrated, are indicated to determine more precisely the limits and other characteristics of the toxic zone under various conditions.

SUMMARY

Copper paint surfaces prevent the establishment of *Bugula neritina* (1) by repelling or killing the larvae and (2) by inhibiting growth and metamorphosis of attached larvae.

Copper paints with leaching rates less than 10 micrograms of copper per square centimeter per day permit the larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 15 micrograms per square centimeter per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms per square centimeter per day, but these do not develop colonies.

No consistent effect of slime film on larval attachment was noted. Its presence is not prerequisite to attachment.

Precise relationships between copper concentration and growth of *Bugula* ancestrulae are demonstrated. Growth in sea-water solutions of copper is inversely proportional to the concentration up to 0.3 mg. per liter. Higher concentrations completely inhibit growth.

The critical copper concentrations affecting various stages of the early life cycle of *Bugula* are as follows: (1) Concentrations greater than 0.3 mg. per liter kill larvae and completely inhibit growth of attached forms, (2) concentrations between 0.2 and 0.3 mg. per liter retard growth and prevent polypide formation, and (3) concentrations less than 0.2 mg. per liter retard growth and polypide development.

No stimulation of growth by copper solutions was observed. There was some evidence that small concentrations of copper stimulated attachment of larvae.

Bugula ancestrulae can recover and develop almost normally after seven days exposure to sublethal concentrations of copper. They can recover after repeated immersions in copper solutions that practically prevent growth. Length of exposure has relatively little effect on their ability to recover from copper poisoning.

A gradient of toxicity extending outward a few millimeters from a copper paint surface is demonstrated.

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A NEW GRAPHIC METHOD OF DESCRIBING THE GROWTH OF ANIMALS

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Growth curves, when conventionally plotted as length on age, are difficult to compare and classify. Moreover, the usual mathematical methods of fitting them, such as, the logistic and the Gompertz are rather laborious and inconvenient for application to large numbers of individuals.

Fortunately, for many purposes, it is unnecessary to describe the whole growth curve; for the part below the inflection point is completed early and the part above the inflection point—the "self-inhibiting" phase, covers the period of life when differences in growth are likely to be most striking. That phase of the growth curve can be appropriately represented by a straight line, the characteristics of which can be treated statistically, by the following graphic method:

Using arithmetic graph paper, with body length represented along both the x axis and along the y axis, plot length at ages 1, 2, 3, 4, $5 \cdots n$ on the x axis against length at ages 2, 3, 4, 5, $6 \cdots n+1$, respectively, on the y axis. For several species on which I have found published length data, these points fall along a straight line. This line can be regarded as a sort of *transformation* of the usual growth curve, and in the following discussion I will call it that.

The nine examples given in Figures 1–3 are based on average lengths of large samples. When lengths of individual specimens are plotted by this method, a straight-line relationship is still obvious, though the points deviate more widely from the line than when averages are used. These deviations doubtless result from several causes, among which random variations in environmental experience and errors of observation readily suggest themselves. For a few species the published growth data failed to produce a straight line. In these cases, the course of growth may differ from that in other animals; or the observed anomalies may reflect some artifactual effect in the data.

Among those species for which this "transformation" results in a straight line, the growth increments corresponding to equal time intervals, for example, between years of age ($l_2 - l_1$, $l_3 - l_2$, $l_4 - l_3, \cdots l_n - l_{n-1}$), have the following interrelations; where l_n refers to the length at any given age, i.e., at the end of any given time interval:[2]

$$\frac{l_3 - l_2}{l_2 - l_1} = \frac{l_4 - l_3}{l_3 - l_2} = \frac{l_5 - l_4}{l_4 - l_3} \cdots = \frac{l_n - l_{n-1}}{l_{n-1} - l_{n-2}} = k$$

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[1] For advice and assistance in the mathematics of this paper, I am indebted to Professors George Polya and Harold Bacon of Stanford University. I am also grateful to my colleagues, Mr. O. Elton Sette and Dr. Frances Felin, for their constant interest and help.

[2] In this discussion, l_1 is not necessarily the value directly obtained by measurement, but a value calculated on the basis of all measurements (*see* Fig. 4).

or

$$l_n - l_{n-1} = k\,(l_{n-1} - l_{n-2}). \tag{1}$$

It is interesting to note that, with $l_0 = 0$,

$$\begin{aligned}
l_2 - l_1 &= k\,(l_1 - l_0) = k\,l_1\\
l_3 - l_2 &= k\,(l_2 - l_1) = k\,k\,l_1 = k^2\,l_1\\
l_4 - l_3 &= k\,(l_3 - l_2) = k\,k^2\,l_1 = k^3\,l_1\\
l_5 - l_4 &= k\,(l_4 - l_3) = k\,k^3\,l_1 = k^4\,l_1\\
&\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\quad\cdot\\
l_n - l_{n-1} &= k\,(l_{n-1} - l_{n-2}) = k\,k^{n-2}\,l_1 = k^{n-1}\,l_1.
\end{aligned} \tag{2}$$

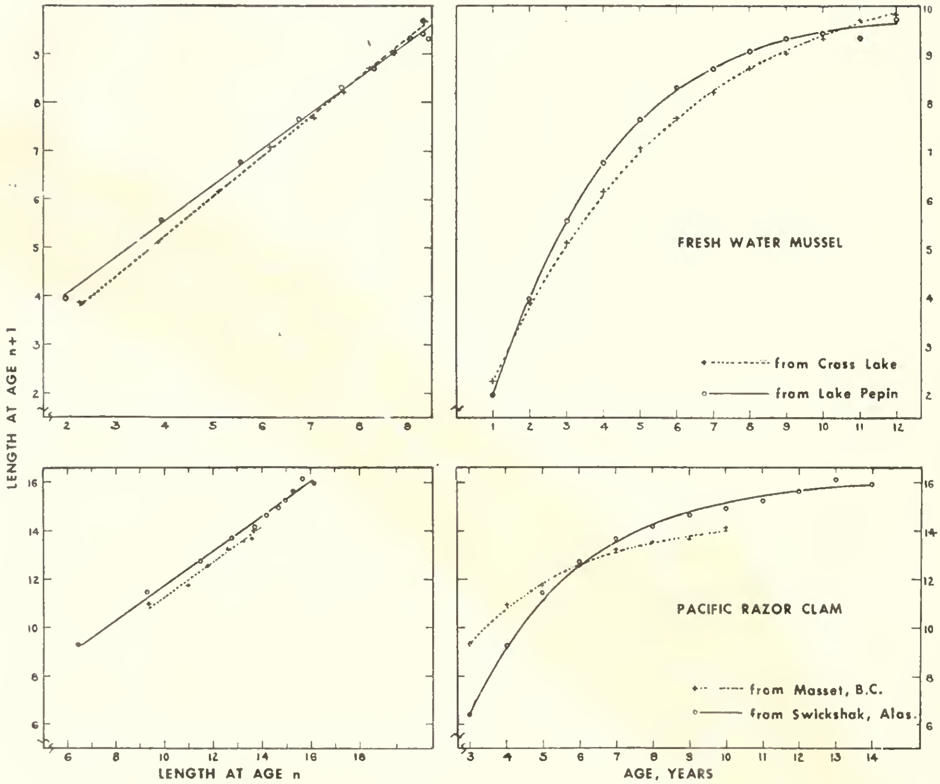

FIGURE 1. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on fresh water mussel from Chamberlain (1931); on Pacific razor clam from Weymouth (1931).

The constant k is positive and less than one; that is, the yearly growth increments decrease.

These relationships are consistent with there being a growth capacity, which is approached from the inflection point at a constant percentage rate. This is in accord with interpretations made by a number of students of growth, among

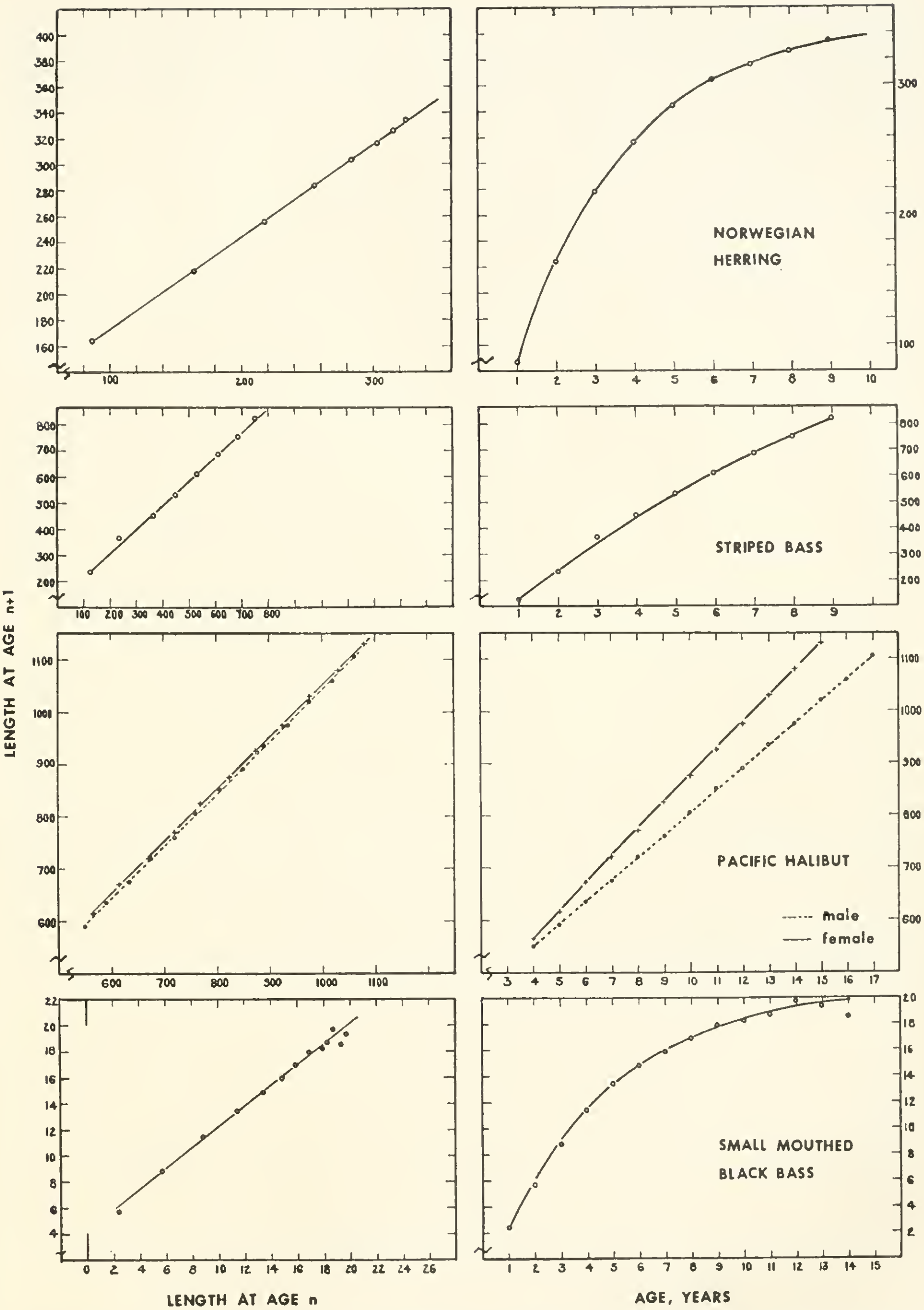

FIGURE 2. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on Norwegian herring from Runnström (1936); on striped bass from Merriman (1941); on Pacific halibut from Thompson and Bell (1934); on small mouthed black bass from Bennett (1938).

them Minot (1908), Wright (1926), Brody (1927 a, b, c), and Weymouth, McMillan, and Rich (1931).

The length at infinite age, l_∞, which can be regarded as the ultimate length or limiting length, can be calculated as follows: The length l_n is attained by adding to l_1 the successive increments,

$$l_n = l_1 + (l_2 - l_1) + (l_3 - l_2) + (l_4 - l_3) + \cdots + (l_n - l_{n-1}).$$

Yet these increments were expressed in the formulas following (1), thus:

$$l_2 - l_1 = k\,l_1,\ l_3 - l_2 = k^2\,l_1, \cdots l_n - l_{n-1} = k^{n-1}\,l_n. \tag{3}$$

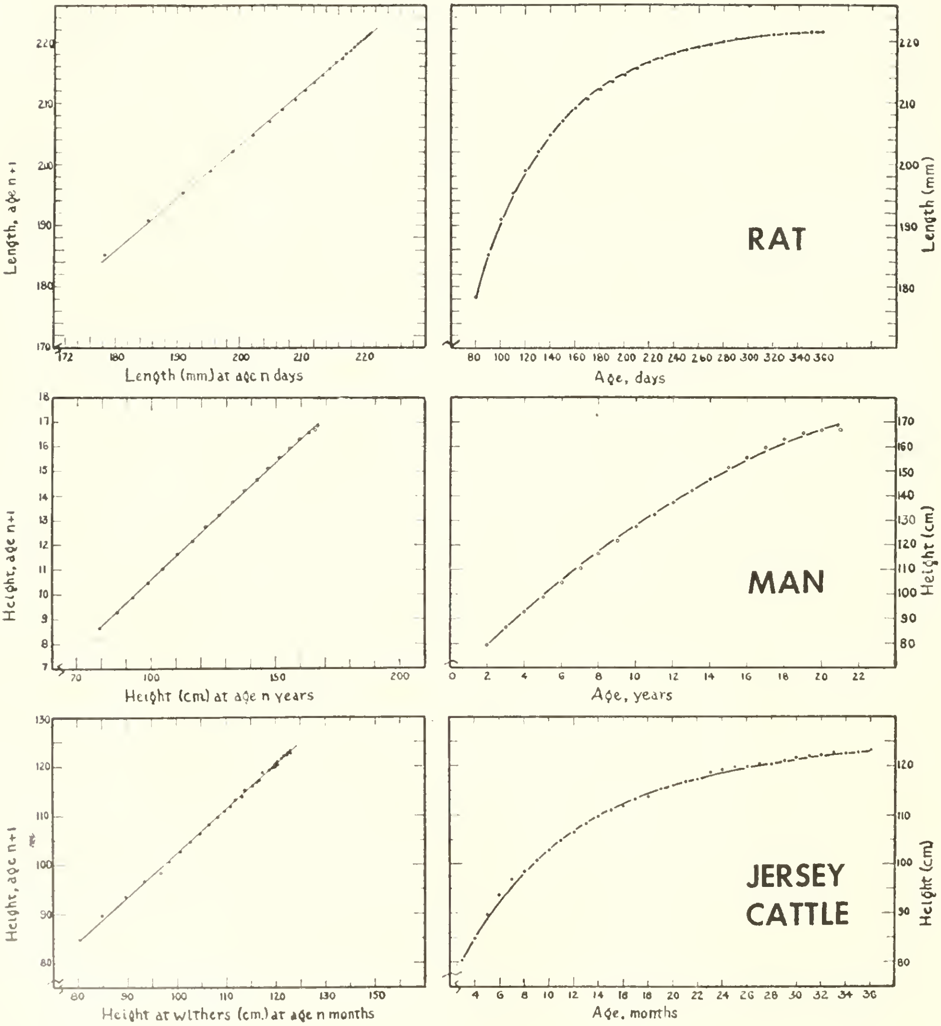

FIGURE 3. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on rat from Donaldson (1931); on man from Thompson (1942); on Jersey cattle from Ragsdale, Elting, and Brody (1926).

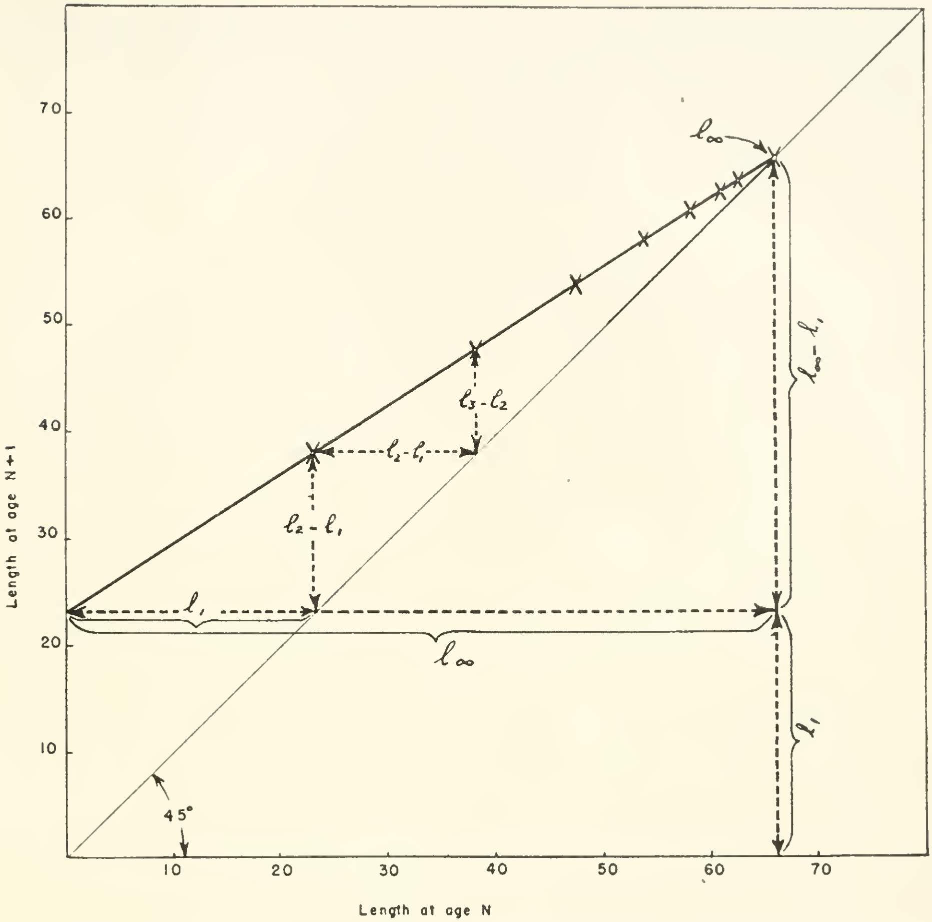

FIGURE 4. Transformation of the growth curve of a hypothetical animal, drawn in a heavy line to illustrate the dimensions given in the text. Note that the ultimate length, l_∞, can be located graphically as the point where the length at age n equals the length at age $n + 1$; also where the transformation intersects a line drawn at 45° through the zero point.

Therefore,

$$l_n = l_1 + k\,l_1 + k^2\,l_1 + k^3\,l_1 + \cdots + k^{n-1}\,l_1 = l_1\frac{1 - k^n}{1 - k} \qquad (4)$$

by the well-known formula for the geometric series. Therefore, when n approaches ∞, l_n tends to l_∞, and k^n to 0, and so we obtain

$$l_\infty = \frac{l_1}{1 - k}. \qquad (5)^3$$

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[3] Where this graphical representation gives precisely a straight line, the above calculation shows that l_n is expressed by the formula $l_n = l_\infty(1 - k^n)$. It is a modification of the "Modified Exponential" (cf., Croxton and Cowden, 1940, pp. 441 ff.), but contains one less parameter, and of course applies only to that segment of the growth curve above the inflection point.

Thus the limiting length l_∞ may be computed readily from the y intercept, l_1 and the slope of the fitted straight line, k; and k, in turn, is readily calculated from any of the ratios preceding equation (1).

According to the series of equations (1) to (4) the slope of the transformed growth curve, k, is the constant given not only by the ratio $\frac{l_n - l_{n-1}}{l_{n-1} - l_{n-2}} = k$ but also by the ratio $\frac{l_\infty - l_{n+1}}{l_\infty - l_n} = k$.

In other words, the amount of growth which remains unfulfilled at the beginning of any time interval, is a constant percentage of the amount of it which had remained at the beginning of the preceding time interval. Consequently the higher the k value, the more slowly growth approaches the limiting length.

This method of plotting growth data permits an objective determination of the limiting length, l_∞, even before that length is "attained."[4] It provides two growth characteristics, k and l_∞, from which the upper segment of the length-on-time growth curve can be reproduced. These constants are so simply derived, that it is practical to determine them for large numbers of individuals. They can be used for studying growth variation within and between populations, hence for distinguishing between races of animals with differing growth patterns. The method is particularly useful in studies of fishes whose scales bear annual rings, from which the growth history of individual specimens can be estimated.

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[4] Strictly speaking, of course, under the terms of this description the limiting length is only approached; it is never attained.

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INVESTIGATION ON THE LOCUS OF ACTION OF DDT IN FLIES (DROSOPHILA)

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Introduction

The experiments reported herein were designed to gain information as to where DDT produces its poisonous effect in the insect.

DDT poisoning in insects is characterized by symptoms of hyperactivity and discoordination of neuromuscular system, followed by convulsions and terminating in death. Isolated legs of DDT treated roaches continue to twitch after complete separation from the body, and isolated legs of normal roaches were induced to twitch by the application of DDT to the cut surface, although they remained quiescent if DDT was not applied (Yeager and Munson, 1945). Likewise the isolated legs of adult blowflies (*Phormia regina*) showed twitching movements when dipped before or after they were cut off from the animal into a one per cent DDT acetone solution, while untreated isolated legs remained motionless (Chadwick, 1945). From somewhat different experiments, Tobias *et al.* (1945) working with roaches suggested that the thoracic ganglia were the critical loci for the action of DDT. From these observations it appeared likely that the symptoms of DDT poisoning in insects resulted from an effect on the central nervous system, but that there existed also peripheral components which were not as yet exactly delimited.

Material and Methods

The experiments were performed on the larvae and adults of the fruit-fly (*Drosophila virilis*). The DDT preparation used was in form of an emulsion, (one per cent DDT, one per cent lecithin, ten per cent peanut oil, emulsified in a 0.95 per cent NaCl solution). This emulsion was injected by means of a micropipet into the abdominal cavity of the insect. The physiological saline solution used throughout the investigation was a Ringer solution modified for Drosophila (H_2O, 1000 cc.; NaCl, 7.5 gm.; KCl, 0.35 gm.; $CaCl_2$, 0.21 gm.). The various concentrations of phenobarbital used were also always made up in this Ringer solution. Imaginal discs were transplanted with the usual Drosophila transplantation technique.

Experimental

Behavior of larvae and adults after poisoning

When DDT emulsion was injected into the abdomen of adult flies which had been slightly narcotized with ether, the response to the poison was immediate. Legs and wings at once went into violent, uncoordinated movements. About twenty seconds after the injection the abdomen, previously motionless, began to convulse; its movements were a rapid succession of short, uncoordinated spasmodic twitches. At first the convulsions were strong and a great deal of the injected emulsion was thus pressed out through the puncture wound. About five minutes after the injection the legs and wings went into a spasm and took up a characteristic position. The legs were drawn toward the body and crossed over ventrally, while the wings were folded backward. This position was maintained until the animal died. The contractions of the abdomen continued for about four to seven hours but became gradually weaker. After seven hours the animal was apparently dead. Although during all of this time the legs and wings remained in their spasmodic condition, one occasionally observed slight twitches of the tarsal segments and of the antennae; the wings, however, showed no movement. It was thus clear that the muscular response to the poison varied in different regions of the body, for the wing and leg muscles soon went into contraction and remained that way, while the muscles of the abdominal wall continued to convulse for a long period.

When an emulsion prepared in the same manner, but containing no DDT, was injected into flies, no effect was noted. The animals recovered from narcosis in the usual way, and were still alive the following day without any apparent injurious effects. Thus the symptoms described above were due to DDT and were not caused by the emulsion itself.

Larvae narcotized with ether were motionless, except for the pulsating heart tube visible through the transparent skin. When such larvae were injected with DDT emulsion, one observed at first a great acceleration of the heart-beat. Convulsions of the body wall began about twenty seconds after the injection. It was difficult to observe the heart-beat while the convulsions were in progress, but it was found in ligatured larvae, which will be described below, that the heart-beat soon became normal again after the initial acceleration. The larval convulsions were very strong and uncoordinated. Contraction wave after contraction wave passed over the creature, somewhat resembling crawling movements, yet the animal was unable to move from its place. The forward and backward movements were much more rapid than the normal crawling movements. Moreover, the animal never extended to its full length but remained partly contracted all the time. Short twitching contractions occurred in various parts of the body, and broke the wave-like contraction into a complex, uncoordinated movement. The larvae moved continuously in this way, some of them for twenty or more hours. The majority of such larvae died within ten hours, but some of them lived for twenty-thirty hours after the injection. The symptoms were the same, whether last (3rd) or late 2nd instar larvae were used for the experiments.

In control experiments, where emulsion containing no DDT was injected, no such symptoms occurred.

Ether Narcosis and DDT Symptoms

Normal flies etherized only until their movements stopped were completely relaxed if removed at that time from the ether. Their wings were in normal resting position and their legs were bent in the way assumed by flies at rest. Such flies recovered about one half hour after their removal from the anaesthesia and then seemingly behaved normally. Yet flies left for a longer time in the etherizer behaved quite differently. Their wings were folded back and their legs were stretched out and held stiffly away from the body. Usually flies thus over-etherized did not recover from the narcosis and died in that position.

The reaction of these differently etherized flies to DDT poisoning was quite interesting. The slightly etherized individuals showed, after DDT injection, the typical DDT symptoms described in the foregoing section. On the other hand, when over-narcotized flies were injected with DDT, one noticed that their legs and wings did not respond to the poison. Since such flies never recovered from the narcosis it might be thought that they were already dead at the time of the DDT injection. This, however, was not the case, for their abdomens showed the typical DDT convulsion. These convulsions continued for about three hours, but were somewhat weaker than those of the slightly etherized animals. About that time the uninjected but over-etherized control animals were still completely immobile and apparently dead. In comparing the uninjected and injected flies, one gained the impression that the DDT treatment in some way partly released the ether block.

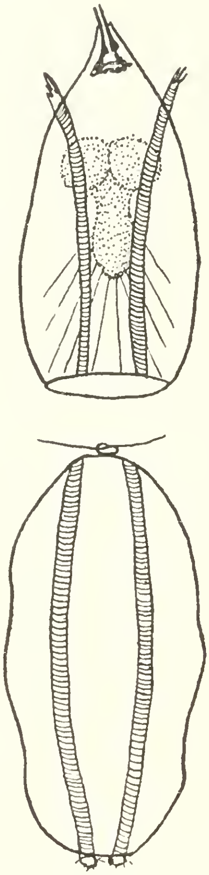

FIGURE 1. Diagram illustrating larval ligature experiments for separating the central nervous system from the rear part of the body. Note location of central nervous system (stippled) in anterior part.

The Importance of the Central Nervous System

The central nervous system of Drosophila is concentrated in the anterior part of the body. In the larva the central nervous system is located in the third thoracic and the first abdominal segments; it consists of the two brain hemispheres, to which is attached a large ganglionic mass (Figs. 1 and 3). This large ganglion is a compound structure, for it includes the sub-oesophageal ganglion, the three thoracic ganglia, and the eight abdominal ganglia. In the adult insect the three thoracic ganglia are separated, but all eight abdominal ganglia unite into one ganglionic mass which extends into the first segment of the abdomen (Fig. 2).

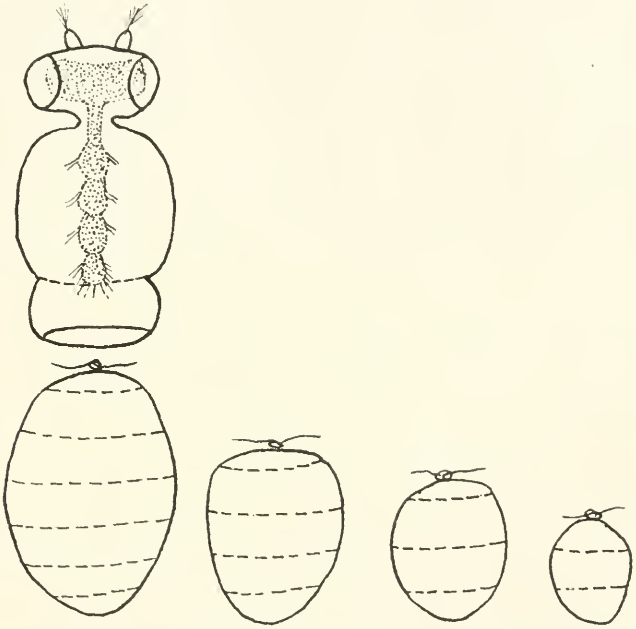

Figure 2. Diagram illustrating ligature experiments on adult flies, showing the separation of progressively smaller abdominal parts from the anterior region of the animal. Note location of central nervous system in anterior part of fly.

The described topography allowed one to separate experimentally large parts of the insect body from its central nervous system. The separation was accomplished by means of a fine silk ligature tied around the body of the animal. Depending upon the position of the ligature, smaller or larger parts of larvae and adults were thus isolated. In all experiments of this kind, the part in front of the ligature was cut away, in order to be sure that no connection to the ganglia remained (Figs. 1 and 2). Abdominal parts isolated in this way were completely motionless and stayed alive for several days.

The most useful symptom for the experimental approach was the contraction of the abdominal wall muscles under the influence of DDT. This effect was very uniform, clearly observable, and lasted a considerable time. The question then arose: were these movements under the control of the central nervous system?

In other words, was the muscular activity caused by an effect of DDT on the central nervous system? To test this possibility, isolated portions of larval and adult abdomens containing no ganglia were injected with DDT emulsion. The response to the injection was called forth immediately. Both larval as well as adult abdomens exhibited the same typical movements that were observed in the abdomens of injected intact flies. Again, as in the intact animals, it was found that the isolated adult abdomen responded somewhat faster than the isolated larval abdomen. That is, the larval abdomen responded at about thirty-sixty seconds and the adult abdomen about twenty seconds after the injection. The DDT convulsions of the isolated parts continued for a considerable time, but not as long as in the intact animals. In the isolated adult abdomen the convulsions clearly became weaker two hours after injection, yet weak contractions were observed five hours after the injection. In the isolated larval abdomens the contractions were still strong four hours after the injection, yet these parts never survived for twelve hours as injected whole larvae commonly did.

A similar sequence of events was observed when only the distal two or three segments of the adult abdomen were isolated from the rest of the body and then injected with DDT emulsion (Fig. 2) or when only two or three segments from the middle of the larval abdomen were isolated by means of two ligatures and then injected.

These findings showed that the central nervous system did not control the abdominal symptoms produced by DDT, since they occurred also in the absence of the central nervous system. However, it must be recognized that this does not imply that the central nervous system was unaffected by DDT.

Body Fragments and DDT Symptoms

In order to reduce the structural complexity of the insect body, in an effort to localize more closely the site of action of DDT, the following fragmentation experiments were performed. Rectangular pieces of skin were cut from the dorsal, lateral, or ventral wall of adult flies and placed in a drop of saline solution. These pieces, about two segments wide, included some muscles of the body wall, fat tissue, tracheae, and nerves but no ganglia. The pieces of dorsal body wall included also part of the heart tube and some alary muscles. In saline solution these pieces remained motionless, but if a few drops of DDT emulsion were added, the isolated muscles in the piece began to twitch. This response began about thirty seconds after DDT was added and continued for about two or three minutes. In control experiments where emulsion containing no DDT was added, no such response occurred.

These experiments confirmed those above by showing that the central nervous system was not necessary for the response of the abdominal muscles to DDT. It would appear that DDT affected either the muscles directly or the peripheral nerves. One way of distinguishing between these two possibilities would be to test a nerve-free muscle preparation with DDT. Technically, however, it is impossible to obtain such a preparation. Other methods were sought to settle this question and are described below.

Treatment With Phenobarbital

Phenobarbital is a drug which depresses central nervous activity in vertebrates. The effect of this substance on flies has not been described previously. When a ten per cent phenobarbital saline solution was injected into the abdomen of adult flies the animals were apparently killed instantly. The slight vibrating movements characteristic of lightly etherized flies stopped immediately after the injection. All of the muscles seemed to relax and the legs and wings were held in normal position.

Flies which had just come out of ether narcosis but were still sluggish lost their coordination when injected with a one per cent phenobarbital solution. Wings, legs, and abdomen moved uncoordinatedly for several hours until the animal finally died. The movement of the abdomen, also uncoordinated, was very different from the symptoms produced by DDT.

Injection of 0.1 per cent phenobarbital solution into adult flies induced narcosis, followed by complete recovery of the animal. One hour after the injection some flies crawled about slowly, while others were still unable to hold themselves up and fell over from time to time. By this time the effect of the ether had worn off and these symptoms were regarded as phenobarbital effects. One-half hour later the coordination of the animals had improved but their movements were still slow. But the next day the animals had recovered and behaved normally.

That phenobarbital affects the nerves rather than the muscles in flies was indicated by the following experiments. Fully grown larvae were split open along the mid-dorsal line. The intestine, Malipighian tubes, fat body and the main tracheae were then removed. Care was taken not to disturb the brain and ganglia in the anterior part of the body. This manipulation was carried out in physiological salt solution. The skin with its muscular layer and the adhering nervous system was placed in a small dish with a wax bottom and covered with fresh physiological salt solution. The preparation was then stretched out and by means of fine pins was fastened to the wax bottom of the culture dish, as shown in Figure 3. In this condition the tissues stayed alive for several hours. From time to time the muscles contracted, showing the typical wave-like contraction pattern of a crawling larva. After these movements had been observed for ten minutes the physiological solution was removed and replaced by a one per cent phenobarbital solution. The movements in the preparation stopped immediately. Again, after about ten minutes during which time no movements occurred the phenobarbital solution was removed and replaced by physiological saline. This solution was in quick succession drawn off and replaced about two or three times. The preparation was thus washed clean of any phenobarbital. It was now observed that normal movements had returned.

Ten minutes later the physiological solution was removed and replaced by one per cent phenobarbital solution; all movements again stopped. When washed and placed in physiological solution again the movements in the preparation reappeared. This procedure was repeated three or four times at intervals of about ten minutes and still the tissues continued to contract spontaneously when in physiological solution.

These experiments indicate that the one per cent phenobarbital solution was apparently not injurious to the tissues for the length of exposure tested. Moreover, while in phenobarbital, no muscular movement was observed, yet if the muscles were stimulated directly by touching them with a fine needle, localized contraction in the region of the stimulus was noted. Following the mechanical stimulus the activated muscles contracted rather rapidly, stayed in the contracted state for some time and relaxed very slowly. This localized response of the muscles to mechanical stimuli when under phenobarbital indicates that the drug had not paralyzed the muscles directly but rather the nerves.

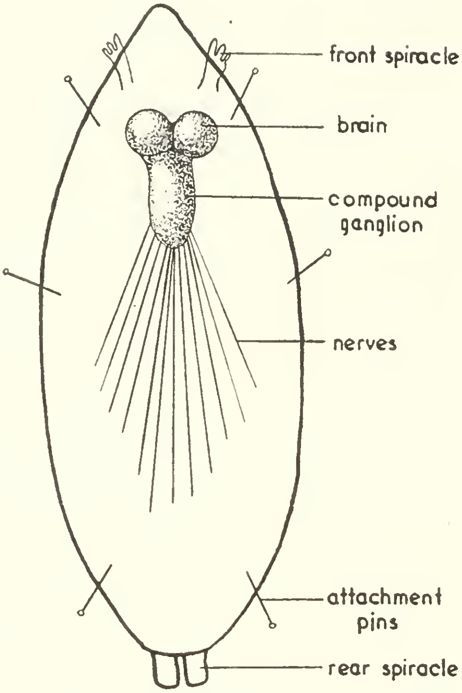

FIGURE 3. Diagrammatic representation of the skin muscle preparation of a whole larva, pinned to the wax bottom of the culture dish.

COMBINED PHENOBARBITAL AND DDT TREATMENT

The knowledge that phenobarbital apparently acted on the nerves but not on the muscles provided a tool for determining whether DDT affects the nervous tissue or the muscles. If DDT could affect the muscles directly DDT symptoms should be provoked in animals paralyzed by phenobarbital. If, on the other hand, DDT affects only the nerves, no DDT symptoms should occur in animals treated with phenobarbital. That the latter is the case was shown by the following experiments.

In physiological solution the muscle preparation (Fig. 3) showed spontaneous crawling movements as described above. If a small amount of DDT emulsion was dropped onto such a preparation, the rhythm of the movements was interrupted; it became uncoordinated rapidly and resembled the movements observed in DDT-treated larval abdomens. If, on the other hand, DDT emulsion was dropped in the same manner onto preparations kept in one per cent phenobarbital solution, no movement whatsoever took place. Moreover, if the physiological solution containing DDT emulsion was washed off from the actively moving preparation, and was replaced by one per cent phenobarbital solution, the movements ceased immediately. However, it was impossible to bring the preparation once treated with DDT back to its normal way of movement by washing the DDT solution off and replacing it with pure physiological solution. Such preparations still showed DDT symptoms. Apparently it was impossible to wash all the DDT out by the methods used. The DDT symptoms were of course stopped by placing the preparation again into phenobarbital.

Similar results were obtained when DDT-treated larval or adult abdominal parts were injected with one per cent phenobarbital solution. The DDT symptoms of such parts ceased immediately after phenobarbital was administered by injection. The reciprocal experiment, where phenobarbital was injected first, yielded the same results, for when DDT was injected into such treated abdomens no DDT symptoms occurred. The symptoms of whole larvae or adults which had been treated with DDT were also stopped immediately by injecting a one per cent phenobarbital solution.

Certainly the effects of phenobarbital on isolated, DDT-treated abdomens indicate that the absence of the central nervous system did not limit the action of the drug, showing that in insects phenobarbital may act on the peripheral nerves.

At this point the discussion of the phenobarbital effects on DDT poisoning must be augmented by an experiment showing how phenobarbital in weaker concentrations caused at least a partial recovery from DDT poisoning. It has been stated before that the legs and wings of adult flies injected with DDT were completely paralyzed five minutes after the injection. Now, when 0.1 per cent phenobarbital solution was injected into such animals, the movements of their legs were restored. These movements were uncoordinated and were similar to those observed in the beginning stages of DDT poisoning before the organism went into spasm. Also, the wings were able to move somewhat and were not folded backward. Even the convulsions of the abdomen were much less pronounced. Leg movements continued for about two hours, which of course was a much longer time than ever noted in animals injected with DDT only. These findings clearly show the antagonistic effect of phenobarbital on DDT.

Capacity of Different Tissues for Growth and Differentiation After DDT Poisoning

If it is true, as the experiments seemed to indicate, that DDT affects only the nervous system, one might expect other tissues to be largely unharmed by the DDT treatment. This expectation can be tested experimentally. It is known (Bodenstein, 1943) that larval tissues will grow and differentiate normally when transplanted into the abdomen of larval or adult flies. In the larvae the transplanted tissues will develop in synchrony with their hosts to imaginal completion, and in adult flies, the transplant will undergo a considerable amount of growth. The capacity of the tissue for growth and differentiation, it is believed, offers a good criterion for testing the condition the tissue is in after being exposed to the poison. Tissues affected by DDT should certainly not develop normally. The following experiments were designed to clarify this issue.

Fifteen last-instar larvae were injected with DDT and placed on moist filter paper. Twenty-two hours later four larvae were still alive and showed typical DDT symptoms. Three of these larvae were opened and their eye discs, leg discs, and antennal discs dissected out. These discs are the primordia for the future eye, leg, and antenna of the adult fly.

They were transplanted into the abdomen of the mature larvae, one disc to each host. The several hosts carrying the transplants from one donor were kept separated from the hosts carrying the transplants of the other donors.

If the transplanted organs were able to develop normally, they should have been found as fully differentiated organs in the abdominal cavity of their respective hosts after metamorphosis. From the fifteen original hosts comprising the cases of all three series, eleven survived the operation. These animals completed their metamorphosis seven days after the operation and emerged. They were then dissected. Three completely differentiated legs, one eye, and one antenna supplied by the first donor were recovered. Hosts which received transplants from the second donor yielded three legs, two antennae, and two eyes, and from the third donor, three legs. All transplants were fully differentiated. As far as the detailed morphological differentiation of the tested organs was concerned, they were found to be completely normal, and there was no reason to believe that the histological differentiation likewise was not normal.

For another experimental series, five leg discs, dissected from the fourth living larva of the original set of DDT-treated animals, were transplanted into the abdomen of five adult flies. Each host in addition to the leg disc also received two ring glands. This structure is necessary for the continued growth of the transplant, for it furnishes a growth-promoting hormone (Bodenstein, 1943). Three days after the operation one host was killed and the leg disc dissected. It had grown but little. Three other hosts were killed six days after the operation and the transplants dissected. These leg discs had clearly become larger. Finally the last host was killed 24 days after the operation and the disc dissected. In this case the transplant had grown considerably and had reached an advanced state of differentiation. The results of this series of experiments were very similar to those obtained in transplanting normal discs in the same manner (Bodenstein, 1939 and 1943).

In conclusion, these two experimental series show that exposure to DDT for twenty hours in no way affected the capacity of the imaginal tissues for growth and differentiation. Hence these findings are further evidence that the nervous system alone is affected by DDT.

SUMMARY

1. The larvae and adults of *Drosophila virilis* were fatally poisoned by injecting a one per cent DDT emulsion into the abdominal cavity. The poison produced a typical pattern of symptoms.

2. The neuromuscular system of the wings and legs was apparently very sensitive to the poison, for they went into spasm long before the muscles of the abdominal wall. There was also a difference in sensitivity to the poison between the larva and adult, the larva being more resistant to the DDT emulsion.

3. Phenobarbital was found to affect the nervous system. Paralysis by phenobarbital was also produced in the absence of the central ganglia. This shows that the drug also affected the peripheral nerves. Muscles of larvae treated with phenobarbital responded to mechanical stimulation.

4. Since DDT produced no symptoms in animals treated with phenobarbital and since animals treated with DDT lost their DDT symptoms when injected with phenobarbital, it was shown that DDT acted on the nervous system. Moreover, body parts which had been isolated from the central nervous system and then treated with DDT stopped convulsing after phenobarbital administration. This shows that DDT affected the peripheral nerves.

5. The methods used do not allow one to determine what part of the peripheral nervous system might be affected. There are three possibilities. The poison might affect (1) the motor nerves leading to the periphery; (2) the myoneural junctions; (3) the peripheral nerve net. It is however still questionable whether such a nerve net exists in *Drosophila*.

6. The antagonistic effect of phenobarbital on DDT was clearly indicated by the fact that the spasm of the legs and wings in DDT-treated flies was partly relieved by treatment with phenobarbital.

7. The conception that only the nervous system is affected by DDT has been strengthened by the fact that larval organs (imaginal discs) which had been exposed to DDT for twenty hours grew and differentiated normally when transplanted into untreated larvae.

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THE CONTRACTILE VACUOLE AND THE ADJUSTMENT TO CHANGING CONCENTRATION IN FRESH WATER AMOEBAE

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INTRODUCTION

In considering the relationship existing between an organism and its environment, it is necessary to distinguish clearly between the environment of the organism and the environment of the individual cells. The effect of the environment on an organism will depend specifically on the extent to which the cellular environment is controlled by the organism as a whole. As pointed out by Krogh (1939), the osmotic environment of the tissue cells of the vertebrates—the body fluid—is maintained at a very constant level. This is true to a lesser degree among the lower organisms. In the free-living protozoa, the cellular environment is the outside environment of the organism and is controlled little, if at all, by the organism. We find consequently that the individual cells of higher organisms have lost their resistance to osmotic changes while the cells of many of the protozoa, of necessity, have retained the ability to withstand considerable variation in the osmotic environment. It is well known that certain bacteria and molds can withstand enormous osmotic changes—which also have been shown to be true of a considerable number of protozoa (Hopkins, 1938; Butts, 1935; Mast and Hopkins, 1941; and Kitching, 1938).

The nature of the adjustments made by cells capable of withstanding great osmotic changes is not clearly understood. In the literature, the assumption is rather generally made that the osmotic concentration of the cellular contents must be maintained at a fairly constant value in order to be consistent with the metabolic processes. This assumption is made, regardless of the fact that evidence for the constancy of the intracellular osmotic pressure is conspicuously absent, especially for the lower organisms. We find that the prevailing opinion as to the function of the contractile vacuole of the protozoa is that it is an organelle which operates to maintain the intracellular osmotic pressure at a relatively constant level which is higher than that of the environment. This theory maintains that excess water, diffusing through the outer membrane into the hypertonic cell, is extracted, isolated into the vacuole, and thus is eliminated when the vacuole is discharged (Kitching, 1938). However, Mast and Hopkins (1941) have shown that the marine *Amoeba mira* (*Flabellula mira* Schaeffer), which can withstand enormous changes in the

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concentrations of the salts of sea water, does not maintain a constant intracellular osmotic pressure. The intracellular pressure varies with that of the environment. They have shown that, if the amoeba is living in, and is adjusted to a medium, the intracellular pressure is always only slightly higher than that of the environment. Thus, a change in the osmotic pressure of the environment results in a corresponding change in the intracellular pressure.

In view of the fact that *Amoeba mira* is a marine organism and does not form contractile vacuoles even in dilute media, it was considered important to extend the investigations to a fresh water amoeba, which forms and eliminates contractile vacuoles, in order to ascertain whether these forms actually maintain a constant osmotic pressure by means of the action of the contractile vacuoles. The amoeba selected was a fresh water form collected from a swamp near Chicago. A single amoeba was isolated and allowed to multiply on an agar culture, using the methods of Rice (1935). All experiments were performed on the progeny of this amoeba.

Methods and Results

Description of the amoeba

As in the case of most amoebae, it is difficult to decide upon its correct name. Figure 1 is a composite diagram illustrating its structure as observed in fresh water. Dujardin's *Amoeba lacerata* (1841) fits it fairly well. We shall then adopt the name of *Amoeba lacerata* Dujardin. Schaeffer (1926) has placed amoebae of this type in the genus *Mayorella* Schaeffer. Thus, using the system of Schaeffer, the name is *Mayorella lacerata* Dujardin.

Amoeba lacerata is a small amoeba, rounds up readily when disturbed, and in that condition has an average diameter of fifteen micra or a volume of about 1500 cubic micra. It varies from a limacine to a flattened fan shape and has a clear hyaline border (Fig. 1*a*) which is thick anteriorly and very thin posteriorly. A few, fine, pointed, indeterminate pseudopodia may project from the surface—anteriorly, superiorly, and posteriorly. Cytoplasmic granules are numerous—(1) an abundance of small, barely visible granules and (2) larger granules (or vacuoles) which are less numerous and more variable in number and size (Fig. 1*b*). The living nucleus (Fig. 1*d*) is spherical with a central endosome. Food is engulfed into the under surface as the amoeba moves over the food particles. Except in rare cases, food is engulfed with little or no water. Ingested food particles coalesce with clear vacuoles (Fig. 1*c*) of the cytoplasm, and the resulting small food vacuoles coalesce with each other as they do in *Amoeba mira* (Hopkins, 1938) to form large food vacuoles (Fig. 1*f*). These vacuoles are readily stained with Nile blue sulfate and neutral red. Egestion of food vacuoles (Fig. 1*g*) occurs at the posterior tip.

No direct connection or relationship between the food vacuoles and the contractile vacuoles has been observed except that occasionally a food vacuole may be ruptured into the contractile vacuole instead of to the outside. At egestion, the food vacuoles are rarely large and watery. Just before egestion they may be observed to decrease slowly in size until little more than food residues remain. This would indicate a protoplasmic absorption of water from the food vacuoles. It is also interesting to note that at the time when this apparent absorption takes place, the food vacuoles are located posteriorly and in close proximity to the region of contractile vacuole formation which MacLennan (1941) and others have called the nephridioplasm. It is entirely possible that the water leaving the food vacuoles is absorbed into the contractile vacuoles.

The contractile vacuoles

In an amoeba moving forward rapidly, the contractile vacuoles form at the posterior end, as shown in Figure 1*c*. When the individuals are flattened out and feeding actively, it is difficult to distinguish the posterior end unless the location of the contractile vacuoles is accepted as a distinguishing feature of the posterior part of the cell. Observations on the origin of contractile vacuoles in active amoebae have revealed the following interesting facts:

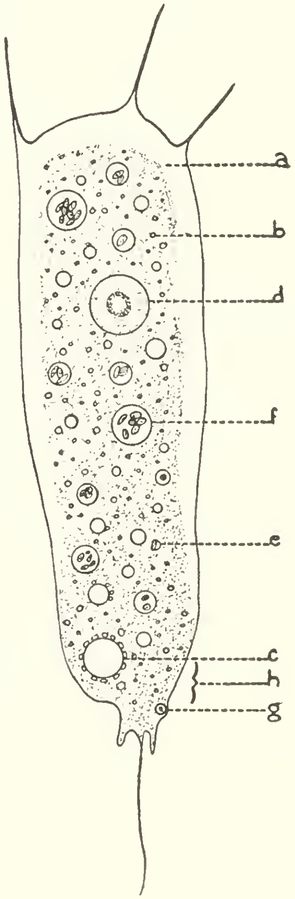

FIGURE 1. Diagram showing the characteristic structure of *Amoeba laccrata* Dujardin. *a*, hyaline border; *b*, large cytoplasmic granule (vacuole); *c*, contractile vacuole; *d*, nucleus; *e*, small clear vacuole adhering to a recently engulfed bacterium; *f*, food vacuole; *g*, food vacuole in position for egestion; *h*, nephridioplasm.

(1) They develop in the posterior third of the cell, or, if they begin to develop in an apparently anterior part of the cell, that part will soon become the posterior part—as evidenced by change in the direction of protoplasmic streaming. If the posterior region of the amoeba becomes separated into two parts by a large food vacuole, contractile vacuoles will arise and be expelled independently on both sides. Pressure on the amoeba by a coverslip will cause contractile vacuoles to arise and be expelled independently in several parts of the cell. This phenomenon was also observed by Howland (1924b) in *Amoeba verrucosa*. A freely moving amoeba, as a rule, has only one formative region for contractile vacuoles.

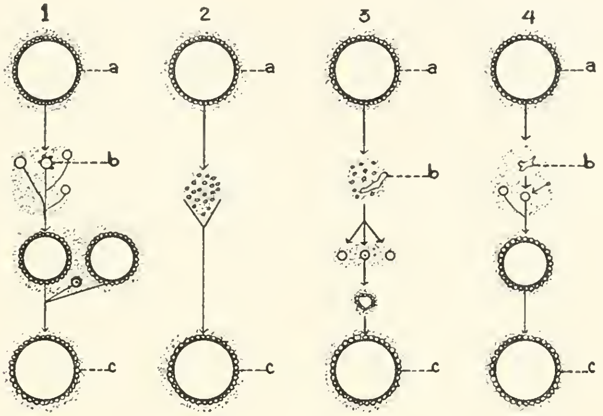

FIGURE 2. Diagrams showing contractile vacuole cycles, *1*, *2*, *3* and *4*. Vacuoles *a*, are expelled to outside leaving residues, *b*, which by coalescence with other vacuoles, small and large, and swelling, form vacuoles, *c*, which are expelled.

(2) The nephridioplasm (Fig. 1*h*) is in the region in which plasmogel is rapidly becoming plasmosol. This region is often under considerable pressure (Mast, 1926). This is evidenced in *Amoeba lacerata* by the observations that the contractile vacuoles are often squeezed into oblong shapes, that food vacuoles and contractile vacuoles which normally never coalesce are sometimes squeezed so tightly together that the food vacuoles rupture violently into the contractile vacuole. The contraction of the contractile vacuole is, however, not always violent. Sometimes the contraction is very slow, while at other times, it may be only partial.

(3) While the contractile vacuoles increase in size by absorption, as will be shown later, the most conspicuous methods of growth are the coalescence of small droplets or vacuoles with one another, of large vacuoles with each other, and of small vacuoles with large vacuoles (Fig. 2). Small vacuoles less than 0.5 micron in diameter—can be observed coalescing with large vacuoles whose diameters are many times greater. Careful focusing on the outer boundary of a large contractile vacuole reveals this phenomenon. The coalescence of two contractile vacuoles is seldom violent. It occurs at about the same speed as the coalescence of two drops of olive oil.

(4) When a vacuole contracts, its contents are expelled to the outside. This occurs usually when the vacuole has a diameter of about 4 micra, but often smaller vacuoles contract, and larger ones may be formed before contraction occurs. Rup-

ture usually takes place through the postero-superior surface of the cell. Sometimes the contracting vacuole leaves no residue, but usually a small residue (Fig. *2b*) remains, which coalesces with other small vacuoles forming new, large vacuoles. The residues are usually spherical, but often may assume oblong, stellate, or other shapes. Whether a residue remains or not, the region from which the previous vacuole was expelled contains small vacuoles which coalesce to form a large vacuole (Fig. *2c*). These small droplets, or vacuoles, are continually appearing in the nephridioplasm. The region is generally devoid of food vacuoles.

The diagrams in Figure 2 illustrate four sequences which have been observed to occur in the nephridioplasm.

(5) All efforts to associate the contractile vacuoles with cytoplasmic granules of any sort have been futile.[2]

Tolerance of Amoeba lacerata for changes in the concentration of the medium

The swamp water from which the amoeba was collected was very dilute. The amoebae were first cultured in water from Lake Michigan to which grains of wheat were added. Attempts to culture it in various dilutions of sea water proved quite successful. It can be transferred, without deleterious effects, directly from lake water cultures to 5 per cent sea water to which grains of wheat are added. Excellent cultures of healthy amoebae develop within five days. Direct transference of the amoebae from 5 per cent sea water cultures to similar cultures made up in various dilutions of sea water results in excellent growth, even when the percentage is as high as 50 per cent. Direct transference to cultures having percentages higher than 50 per cent results for the most part in complete failure or very slow growth. If, however, the amoebae are first cultured in 50 per cent sea water and then transferred to 75 per cent or 100 per cent, survival and growth result. Slow growth may even be obtained in 125 per cent by adjusting the amoebae to 100 per cent and then transferring them to 125 per cent sea water.

The volume of the amoebae and the concentration of the culture medium

A study of the relation between the volume of the amoebae and the concentration of the culture medium in which they were grown has been made. The amoebae for this study were all from the same 5 per cent sea water culture and were inoculated into cultures of varying concentrations from one per cent to 75 per cent sea water. To each culture five sterile grains of wheat were added. Five cultures were set up at each concentration in exactly the same way and at the same time. When abundant growth and reproduction had occurred in all the cultures the amoebae were caused to round up by pipetting the culture fluid back and forth. Their diameters were then measured under the microscope with an eyepiece micrometer. At least 100 amoebae were measured for each concentration. The age of the cultures was the same at the time of measurement. A total of over 1,750 amoebae were measured. The results are presented in Figure 3, in which the average volume is plotted against the concentration of the culture. The increase in volume with an increase in concentration appears significant statistically. (The standard errors are graphically indicated in the curve by the length of the vertical lines drawn through the points.) The total range of variation in average volume is, however, only a little over 750 μ^3. This increase in volume with concentration, if significant, is probably due to a lower rate of fission in respect to growth in higher concentrations than in lower concentrations. It should be mentioned here, however, that much greater variations occur in average volume of amoebae from cultures when the nature of food is allowed to vary more than was the case in this series of cultures. (See average volumes of cultures of Figure 4.)

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[2] The very small vacuoles which form the larger contractile vacuoles undoubtedly have been mistaken for granules by various authors.

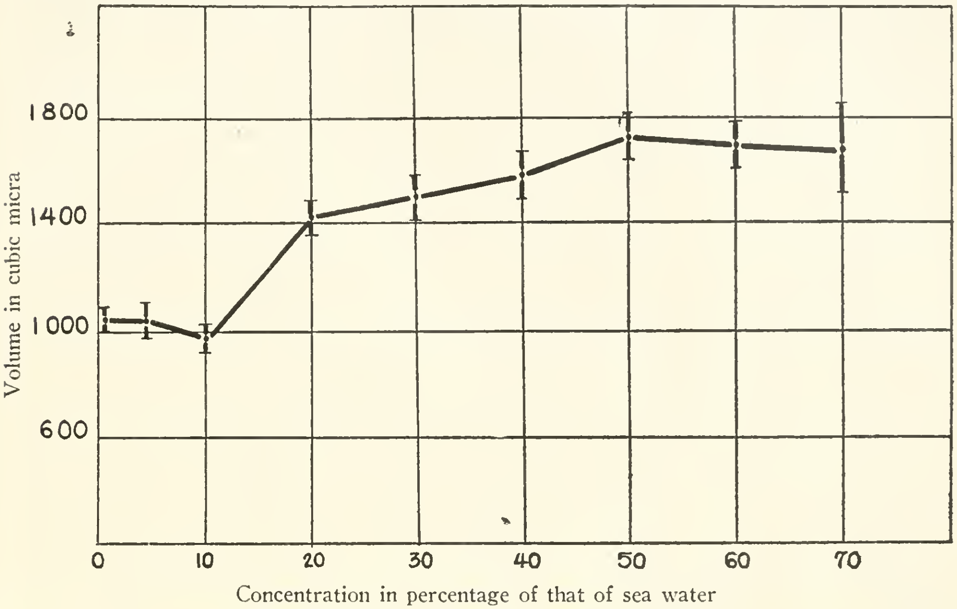


FIGURE 3. Graph showing relation between volume of amoebae and the concentration in which they are grown.

Relation between the protoplasmic osmotic pressure and osmotic pressure of the external medium

In amoebae which have become adjusted to 50 per cent sea water, is the cytoplasmic concentration the same, higher, or lower than that of amoebae adjusted to 5 per cent or 20 per cent sea water? What relation does the cytoplasmic concentration bear to the concentration of the culture medium?

Since these amoebae round up into spheres when agitated, the osmotic concentration of the protoplasm can be readily approximated. When the amoebae are placed in sea water of a given strength with a resulting decrease in volume, we can safely conclude that the osmotic pressure is higher externally than internally. If, on the other hand, the volume of the amoebae increases, the osmotic pressure of the protoplasm is greater than that of the medium. If, however, the plasmalemma is permeable to the solutes of the medium the permanence of the change will depend upon the degree and speed of solute penetration.

The results of experiments designed to answer the above questions were as follows:

Amoebae raised in 5 per cent sea water, and consequently adjusted to it, are placed in one per cent sea water. They immediately swell to a maximum volume and then gradually decrease in volume until approximately their original volume is regained. The smaller the change in concentration, the more rapid is the return to original volume. The same is true when amoebae cultured in any strength in which they will live are placed in higher and lower concentrations.

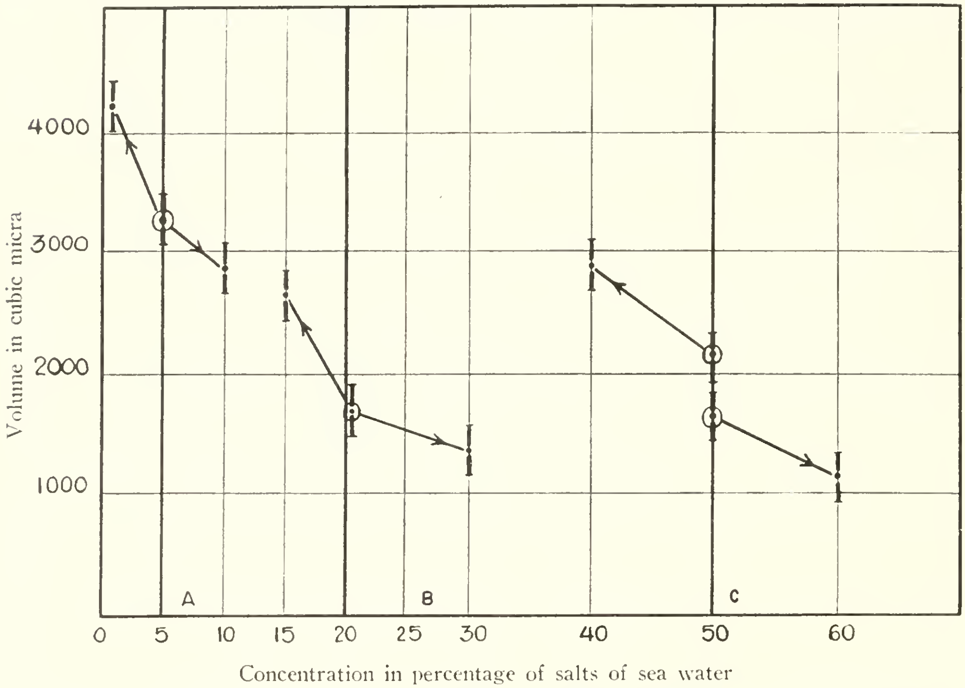


FIGURE 4. Graph showing volume change occurring immediately when amoebae grown in various percentages of sea water are transferred to lower and higher percentages respectively. *A*, amoebae grown in 5 per cent transferred to one per cent and 10 per cent; *B*, grown in 20 per cent and transferred to 15 per cent and 30 per cent; *C*, grown in 50 per cent and transferred to 40 per cent and 60 per cent.

The results of a statistical study of the initial changes in volume when the concentration of the medium is increased or decreased from that of the culture medium are given in Figure 4. In this experiment the amoebae were cultured in 5 per cent, 20 per cent, and 50 per cent sea water (Fig. 4*A*, *B*, and *C* respectively). The amoebae were agitated in the culture; the diameters of from fifty to one hundred individuals measured; the volumes calculated and averaged. The concentration was increased on some and decreased on others; and then the volumes of fifty amoebae whose medium was concentrated, and fifty amoebae whose medium was diluted were measured and averaged. Measurements of volume were all made between 5 and 30 minutes after the concentration was changed. In the figure, the circles give the average volume of the amoebae in culture medium. The other points represent the average volume of amoebae which have been transferred to the concentration indicated. Volume is indicated on the ordinates. The concentration of the culture is indicated on the abscissas. The length of the vertical lines through the points represents the extent of the standard error.

These results indicate very definitely that the osmotic pressure of the protoplasm of *Amoeba lacerata* varies in the same direction as that of the external medium as was found to be the case in *Amoeba mira* (Mast and Hopkins, 1941). The osmotic pressure of protoplasm of amoebae cultured in 5 per cent is not over that of 10 per cent, nor under that of one per cent sea water. The osmotic pressure of those cultured in 20 per cent sea water is not over that of 30 per cent nor under that of 15 per cent. Finally the osmotic pressure of those cultured in 50 per cent sea water is not over that of 60 per cent nor under that of 40 per cent. Due to the rapid return of the amoebae to their original volume when small concentration changes were made, we were unable to make more accurate measurements of protoplasmic concentration. Therefore, we are unable to say definitely whether the adjusted amoebae were isotonic, slightly hypotonic, or hypertonic to the culture medium. Since the amoeba can live as well in 50 per cent sea water as it can in 5 per cent, and since the protoplasm of amoebae living in 50 per cent assumes a concentration greater than that of 40 per cent sea water, a wide range of protoplasmic concentrations appears to be compatible with the metabolic processes.

Factors affecting the activity of the contractile vacuole system

Contractile vacuoles form and are expelled in all concentrations of sea water in which the amoebae will live and reproduce. The rate of formation and expulsion is, of course, slower in concentrated than in dilute culture media. On encystment, growth and expulsion of contractile vacuoles cease and are resumed when excystment occurs. Kitching (1938) has shown that the oxygen tension of the medium is an important factor in the activities of the vacuoles of some protozoa, including various Peritrichs, *Amoeba proteus,* and *Amoeba mira.* During the present experiments, it has been observed that if the amoebae are sealed under a coverslip and left for some time, the rate of vacuolar expulsion is invariably retarded. On the other hand, if fresh aerated culture fluid is continuously circulated under the coverslip, the vacuoles remain active. This would suggest that oxygen is important in the activity of the contractile vacuoles. More precise experiments on the effect of oxygen are necessary.

A study of the effect of the hydrogen ion concentration of the medium was made. Sudden increases and decreases cause an increase in the rate of vacuolar activity. On the other hand, if amoebae are adjusted to and living at a given pH, there is no consistent relation between the pH of the medium and the rate of fluid elimination.

The effect of the concentration of the culture medium on the activity of the contractile vacuoles

An extensive study was made of the relation between the concentration of the culture medium and the rate of fluid elimination by means of the contractile vacuoles. In view of the fact that uncontrollable factors act to affect the activity of the vacuoles, the study had to be statistical. For these experiments amoebae were inoculated into cultures made by adding six grains of wheat to various percentages of sea water in Petri dishes. The rate of elimination was measured only after adjustment evidenced by growth and reproduction was complete. Measurements were made of the rate in amoebae living in 5, 10, 15, 20, 25, and 50 per cent sea water. As we have seen, this amoeba flourishes equally well in all of these concentrations.

The rate of fluid elimination was measured as follows: A coverslip was cleansed and dropped onto the bottom of a culture dish; the culture fluid was agitated with a pipette and the amoebae were allowed to settle and attach themselves onto the coverslip. When attachment had occurred, the coverslip was then inverted and placed on a slide between two parallel streaks of vaseline. The streaks of vaseline served to stabilize the coverslip, prevent the amoebae from being crushed between slide and cover, and allow free circulation of water underneath. During an experiment, fresh aerated sea water of the same strength as the culture medium of the particular amoebae being observed was continuously added at one end of the cover and drawn out at the other by means of a strip of filter paper. Observations were made under an oil immersion lens. The diameters of vacuoles were measured with a micrometer eyepiece and the volumes calculated. The vacuole was always measured just before expulsion. The fluid elimination of an amoeba for a ten minute interval was obtained by adding the volumes of the vacuoles formed during that time. The amoeba was then mechanically agitated, causing it to round up, and its volume obtained by measuring its diameter while thus rounded. The rate was calculated in terms of the cubic micra eliminated per minute by 100 cubic micra of protoplasm.

The results of this study of the rate of elimination of amoebae living and reproducing in various concentrations of sea water are presented in Figure 5. The rates of over one hundred amoebae were measured to obtain the curves in this figure. The rate of each amoeba measured is represented by a point. The average rate of all the amoebae measured in each percentage of sea water is represented by a square, while the highest rate obtained for each concentration of medium is represented by a circle. The average rates have been connected by a curve, as have the maximum rates. A third curve has been drawn whose shape is determined by the formula: $RC = K$, where R is the rate of elimination, C is the concentration of the medium to which amoebae are adjusted, and K is a constant.

If we take into consideration cysts whose rates are zero and which are not included in the figure, we find that the rates of amoebae grown in any concentration may vary from zero to a maximum. The maximum rates vary inversely with the concentration. Neglecting those amoebae, or cysts, whose rates are zero, the average rates also vary inversely with the concentration. Neither the curve for the maximum rates, nor that for the average is exactly parallel with the curve expressed by the equation $RC = K$, but the parallelism in both cases is sufficient to justify the conclusion that under ideal conditions in which concentration is the only variable, the equation would express accurately the relationship existing between the concentration of the medium and the rate of elimination.

Kitching (1938) has pointed out that if a high unalterable protoplasmic concentration was maintained by the action of the contractile vacuoles, the rate of

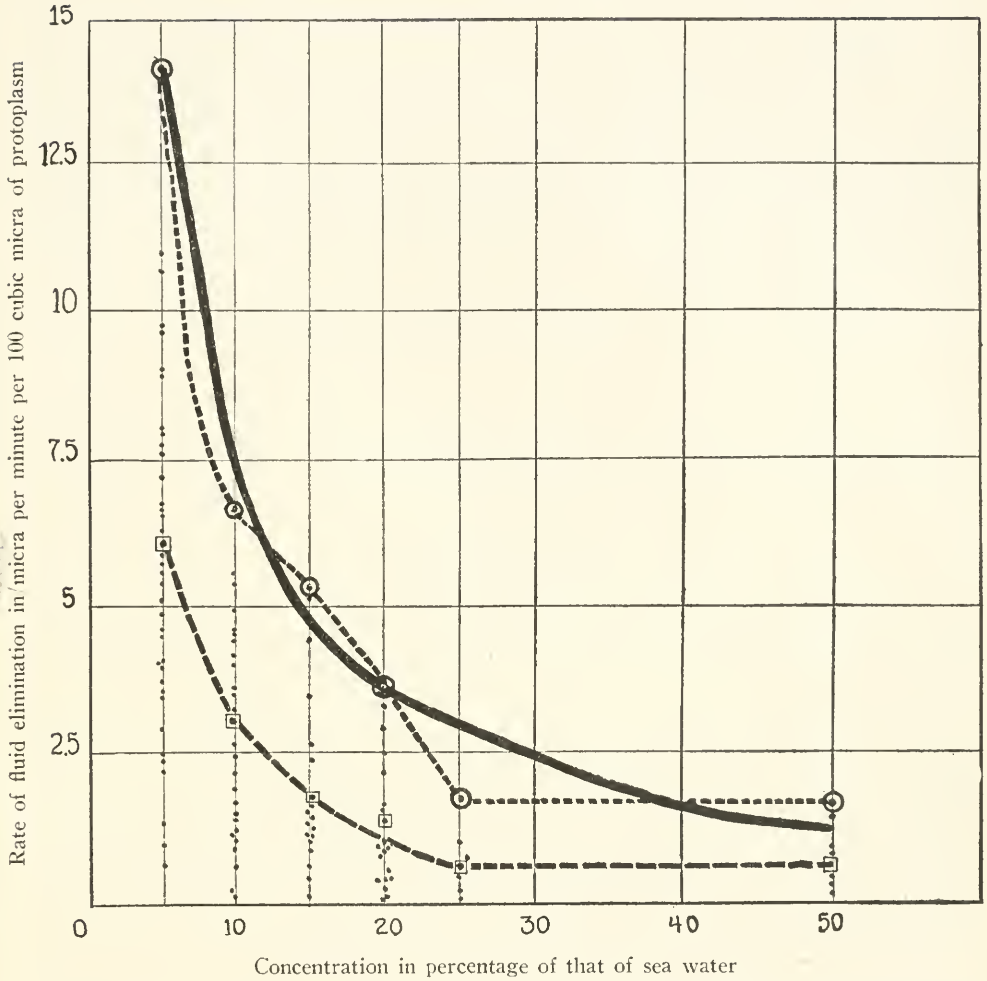


Figure 5. Graph showing the relation of the rate of fluid elimination to the concentration of salts in sea water. The amoebae were tested in the concentration in which they were grown. *Squares,* average rates; *circles,* maximum rates; *dots,* individual rates. *Solid line* is curve for equation; R (rate) × C (concentration) = K (constant).

elimination should be inversely proportional to the external concentration, and that the curve should be a straight line whose slope is constant instead of a curve whose slope increases as the external concentration decreases. The curves obtained by Kitching (1938) for Peritrichs, those obtained by Mast and Hopkins (1941) for *Amoeba mira,* and those obtained here, never approach a straight line. The actual rates are either always too low in the intermediate concentrations or too high in dilute and concentrated solutions, depending of course upon the rate which is assumed to be correct. Therefore, in view of these facts, it is not possible that the correct curve is a straight line.

The effect of changing the external concentration on the volume of individual vacuoles

(A) Vacuoles remaining in the amoeba: When an amoeba with a large contractile vacuole is adjusted to 5 per cent sea water and is placed suddenly in 100 per cent sea water, the amoeba shrinks. By careful observation it can also be observed that the contractile vacuole shrinks. The membrane of a vacuole for a while shows sufficient elasticity to maintain a spherical shape, but the shape is not maintained. As more and more water is drawn out of the vacuole, the membrane collapses (see Fig. 6).

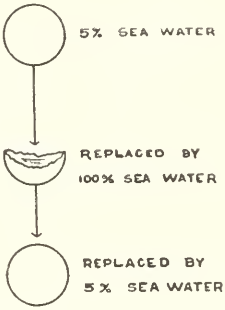


FIGURE 6. Diagram illustrating the collapse of a contractile vacuole when subjected to hypertonic sea water, and its recovery when placed in sea water to which it originally was isotonic.

If the amoeba, now in 100 per cent sea water with the vacuole in the condition shown in Figure 6, is returned to 5 per cent sea water, the amoeba and the collapsed vacuole both absorb water and swell. The vacuole soon regains its original volume and may even become larger. It would appear from this experiment that the vacuole is acting as a simple osmometer. When the hypertonic sea water was added, the water was extracted from the vacuole but not the dissolved substances. Consequently, when dilute sea water was again added this substance acting osmotically drew water back into the vacuole.

(B) Vacuoles removed from the amoeba: In the preceding experiment, it could still be argued that the swelling of the vacuole was due to a secretory process requiring oxygen (Kitching, 1938), since the swelling of the vacuoles took place in the amoeba and in the presence of the cellular activities. If, however, the vacuole was removed from the cell and suspended in the medium free of all contact with protoplasm the influence of other cell parts would be eliminated.

By crushing amoebae under a coverslip while watching the vacuoles closely it was observed that the vacuoles do not always disintegrate; some of them remain intact after they are liberated, free of protoplasm, into the medium. When the vacuoles are thus exposed to the outside medium they swell noticeably, indicating that the elastic forces of the cell were inhibiting swelling to some extent. When such isolated contractile vacuoles from amoebae adjusted to 5 per cent sea water are changed to 100 per cent, they shrink. Then, on being returned to 5 per cent sea water, they swell just as they do while still in the amoebae. *It is obvious then that they act as simple osmometers; that their contents contain osmotically active sub-*

stances in solution, and that the swelling of the vacuoles does not depend upon other protoplasmic processes.

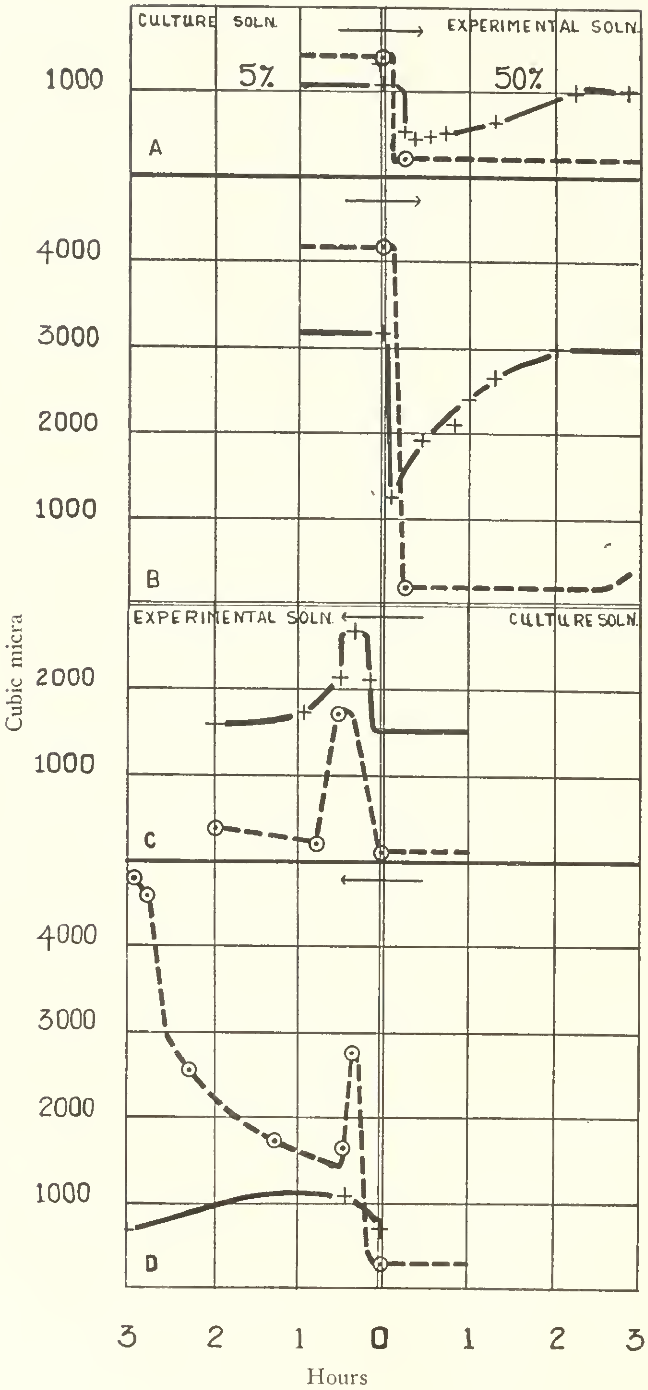
The adjustment of Amoeba lacerata to changing concentration

We have described the immediate effects upon the amoeba and contractile vacuole of changing the concentration of the medium. The story is not completed, however, until we have described the complete series of changes in volume and in the rate of elimination which occur during the adjustment process. Observations have been made upon the volume and vacuolar activities during the adjustment process, both when amoebae cultured in 5 per cent sea water were changed to 50 per cent and when amoebae cultured in 50 per cent sea water were changed to 5 per cent.

(I) Adjustment of amoebae cultured in 5 per cent sea water to 50 per cent sea water: When an actively moving amoeba, raised in 5 per cent sea water, is changed to 50 per cent sea water and allowed to remain, it rounds up and its contractile vacuole shrinks greatly. The amoeba remains for some time in the inactive spherical condition. The contractile vacuole increases in volume very slowly. Repeated measurements of the diameter of the amoeba itself reveals the fact that it increases slowly in volume. This increase continues until it has regained approximately the value it had in 5 per cent sea water. As soon or soon after it has regained this original volume, protoplasmic streaming becomes more definite, attachment to the substratum is made, and normal locomotion begins. The rate of elimination of fluid by the vacuole remains at a low level. In Figure *7A* and *B* are plotted curves showing the observed changes in protoplasmic volume and the rate of elimination of two amoebae when they were suddenly changed from 5 per cent sea water in which they had been cultured to 50 per cent sea water. In these experiments the rate of fluid elimination by the contractile vacuoles was measured, 50 per cent sea water added, and the volume and rate measured again. Since the rate of elimination was so extremely slow it was possible to make only one measurement before adjustment was completed, but the diameter of the amoeba was measured at frequent intervals until adjustment was complete and locomotion resumed.

(II) Adjustment of amoebae cultured in 50 per cent sea water to 5 per cent sea water: In Figure *7C* and *D,* we have plotted curves showing observed changes in volume and the rate of elimination when amoebae were suddenly changed from their 50 per cent sea water culture to 5 per cent sea water. In these curves it will be observed that (1) the amoebae swell and then shrink until their original cultural volume is regained, (2) the rate of elimination increases rapidly to a high value which may either remain high, or after an initial increase may fluctuate, and (3) that the time required for the amoebae to regain their original volume may be long or short.

In this experiment it appears quite possible that the activity of the contractile vacuole may have been helpful in bringing the volume of the amoeba back to normal. But we already know from experiments that when adjustment is complete the amoebae will now shrink if placed in 10 per cent sea water. Therefore, while the activity of the contractile vacuole may have helped the amoebae to regain their


CULTURE SOLN
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3
2
1
0
1
2
3
Hours

Figure 7.

normal volume, the protoplasmic concentration at the same time has dropped from a value approximating that of 50 per cent sea water to a value approximating that of 5 per cent sea water.

Discussion

Krogh (1939) after reviewing the works of various authors on the functions of the contractile vacuoles of the Protozoa, concluded that the evidence was predominantly in favor of the "osmoregulatory theory," which holds that these vacuoles serve to maintain the internal protoplasmic concentration at a constant level which is higher than that of the outside medium. In view, however, of the work of Buchthal and Peterfi (1937) who found only slight potential differences existing between the protoplasm of *Amoeba sphaeronucleus* and the outer medium, Krogh concludes his discussion with a "warning against too schematic conceptions." He says further, "It seems possible that the contractile vacuole may come much nearer in its function to the kidney of higher animals than is indicated by direct studies of osmotic regulation."

The contents of the contractile vacuoles

Weatherby (1927) was unable by means of very delicate tests to show the presence of urea or ammonia in the contractile vacuoles of paramecia. Kitching (1938) points out that the maintenance of a constant concentration higher than the outer medium would require the elimination of fluid more dilute than the protoplasm. He believes from his experience that it is entirely possible that these vacuoles contain only distilled water. Ludwig (1928) argues that carbon dioxide is eliminated by way of the contractile vacuoles, but without evidence. The possibility of the elimination of carbon dioxide, carbonic acid, and carbonates by the contractile vacuoles, however, has not been disproven. Various authors have maintained that granular anlagen give rise to contractile vacuoles (Lloyd and Scarth, 1926; Taylor, 1923; Howland 1924a; MacLennan, 1933). Hopkins (1938) and Mast and Hopkins (1941) maintain that the food vacuoles of the marine amoeba, *Amoeba mira,* in addition to being the seat of digestion, act as do contractile vacuoles and that the anlagen of these vacuoles may contain granules. They also show that the substances that make up these granules may never aggregate in the form of granules but remain in solution in the vacuoles or their anlagen. The present observations on *Amoeba lacerata* do not show definite connections between granules of the cytoplasm and the contractile vacuoles or that their anlagen contain granules, but they do show that they contain osmotically active substances.

The concentration of the protoplasm

The actual concentration of the protoplasm has never been ascertained. When we speak of the osmotic strength or activity of a cell we can of necessity refer only to the attraction of the cell for the solvent and compare the strength of this attrac-

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FIGURE 7. Graphs showing changes in volume and rate of fluid elimination of individual amoebae when they were transferred from the percentage of sea water in which they were grown to lower or higher percentages. *Solid curves,* volume changes; *broken curves,* changes in rate of fluid elimination. Arrows show the direction of the concentration changes. *A* and *B* from 5 per cent to 50 per cent sea water; *C* and *D* from 50 per cent to 5 per cent sea water.

tion to the attraction exerted by known solutions. A cell is said to be isotonic to a solution of known strength when the affinity of the cell and the solution for solvent are equal; i. e., a further increase in the strength of the solution would shrink the cell. No deductions can be made as to the total molecular concentration of the cell components. The osmotic concentration, the intermolecular attractions and repulsions of the protoplasmic molecules and tendencies of the protoplasm to become hydrated are all involved. The tensile or elastic strength of the protoplasm, membranes, and cell wall all work in opposition to the osmotic concentration and hydration. The shrinkage or swelling of a cell depends upon a summation of these inside forces. Most cells are in equilibrium with their environment; i. e., the summation of forces inside equals those outside. The cells forming contractile vacuoles are said to be exceptions according to the osmosis-secretion theory of Kitching (1938). Cells forming contractile vacuoles live in media much more dilute than solutions necessary to plasmolyze them. Such cells must be absorbing water continuously from their more dilute outer medium and continuously bailing out fluid, more dilute than the protoplasm, by the action of the contractile vacuole. Even though the concentration of the protoplasm is higher than the outside medium, this does not follow. The elastic or tensile strength of the membrane and ectoplasm or plasmagel, and for that matter intermolecular attractions of the protoplasmic molecules must exert considerable opposition to the entrance of water. The results here presented and those of Mast and Hopkins (1941) show that *the osmotic activity involved in collecting water eliminated by the contractile and food vacuoles is resident in the vacuoles themselves not in the surrounding cytoplasm.* The surrounding cytoplasm serves merely as a membrane through which the water is drawn. It is also clearly seen from these results that we cannot speak accurately of the osmotic activity of the protoplasm in general. Only of specified parts which have no internal differentiation can we hope to accurately be specific as to osmotic activity.

The concentration of the protoplasm of *Amoeba lacerata* is only slightly different from the surrounding medium. The present experiments demonstrate this fact. (see Figures 4 and 7). The results presented in Figure 5 are consistent only if we assume that the concentration of the protoplasm outside of the vacuoles varies in the same direction as the medium. If we also assume that the protoplasm by oxidative or other metabolic processes gives rise to a constant supply of metabolic by-products for isolation into the vacuoles which swell and coalesce in a constant manner (which is true in the average amoeba), then the curve of Figure 5 is explained perfectly. It is unfortunate that the experiments on the rate of elimination could not be continued below 5 per cent sea water. The reason for this inability was the extreme variability of rates obtained. In other words, in extremely dilute media, the rate of elimination showed little or no dependence upon the osmotic pressure of the external medium. This leads to the conclusion that in extremely dilute media, the osmotic pressure of the external medium is of little consequence in determining the rate of fluid elimination.

The function of the contractile vacuoles

If water is absorbed mainly by the cell due to the osmotic activity of the contents of the contractile vacuoles, the primary function of the vacuoles is not the elimination of water. *The water eliminated by the vacuoles is only water absorbed as a* *consequence of their own contents.* The osmotically active contents of the vacuoles are discharged to the outside and are wasted. These wastes are in all probability metabolic by-products. They could be incompletely oxidized food, carbonic acid, carbonates, ammonia, urea, uric acid, or other waste products.

Kitching (1938) has shown that activities of contractile vacuoles and volumes of various protozoa are controlled by the oxygen tension. He takes this fact to mean that oxidative energy is utilized in extracting water from the protoplasm and the secretion of this water into the vacuoles. He argues that energy is needed since this is accomplished against an osmotic gradient. He also finds that cyanide inhibits the action of the contractile vacuoles in these forms and that coincident with cyanide inhibition, the body volume increases. When cyanide is removed, the contractile vacuoles resume their activity and the cell volume returns to normal. His interpretation of these facts is that the removal of oxygen or the addition of cyanide inhibits oxidation which in turn deprives the vacuoles of energy and they cease extracting water from the protoplasm; but meanwhile the cytoplasm continues to absorb water from the medium thus swelling the cell. He omits any consideration of the effect of cyanide on protoplasm except the inhibition of oxidation. His interpretation does not take into consideration the following possibilities: (1) While the relation of oxygen tension to vacuolar activity is demonstrated, this relation could be as well explained by the formation of osmotically active substances in vacuoles as a result of oxidation. (2) Cessation of the oxidative process may initiate many changes in the protoplasm, for example, its degenerative breakdown which would cause swelling. Kitching's own results, the results of others, and the present results are more consistent with the conclusion that the contractile vacuoles function to eliminate metabolic wastes, and that the elimination of water is merely a consequence of the osmotic activity of these wastes.

When one stops to consider the possibility of the formation of vacuoles more dilute than the protoplasm, it is seen to be an impossibility and contrary to known physico-chemical laws for the following reasons. For it to be possible, water would have to be isolated and separated against solution and chemical forces. This water would have to be isolated into regions of equal hydrostatic pressure by some force which cannot exist since water has been attracted into the protoplasm by exactly opposite forces. There are two ways by which water can be separated from protoplasm, i.e., setting up forces which attract or repulse water more strongly than protoplasm. (1) By chemical changes occurring in regions of the protoplasm, for instance, oxidation which results in localized increases in chemical and osmotic forces (vacuoles). (2) By changes in protoplasm in general. Energy resulting from the oxidation of food can be used in polymerization whereby the forces leading to hydration are lost. This releases water from combination with the protoplasm. The water would tend to collect in localized regions, but during this collection, waste products become dissolved and consequently, the vacuoles so formed would contain waste and salts, not distilled water. Oxidation in the protoplasm enhances the water-combining powers of some substances, but not all.

If the vacuoles contain a solution whose osmotic pressure is less than that of the surrounding cytoplasm, then water will be drawn back into the cytoplasm and not expelled to the outside. This statement, however, would not be true if the vacuole membrane were impermeable to water.

In the experiments concerned with isolated vacuoles, it has been clearly shown that the internal contents of the vacuoles do have an osmotic pressure and that the vacuole membrane is permeable to water. *The osmotic pressure of the vacuoles is either equal to or greater than that of the cytoplasm.*

The relation between the osmotic activity of the protoplasm as a whole and that of the external medium in which the amoebae live

We have demonstrated that the osmotic activity of *Amoeba lacerata* varies directly with that of the environment. This also is true of *Amoeba mira* (Mast and Hopkins, 1941) and of the peritrichs investigated by Mast and Bowen (1945). In addition, it appears that *Amoeba proteus* (Mast and Fowler, 1935), peritrichs (Mast and Bowen, 1945), plant cells (many authors) and many other cells maintain a higher osmotic pressure than their natural medium. This is the cause of turgidity. In the protozoa with contractile vacuoles as well as other animal and plant cells, the extent of this hypertonicity of a cell over its environment must depend upon the strength of the membranes, and other ectoplasmic structures. The external medium remaining constant, some of the factors that would cause swelling of cells are: (1) weakening of cell boundaries, (2) peptization of protoplasmic proteins, (3) increase in cellular anabolism without increase in cellular excretion, or (4) merely failure of the excretory processes.

The extent of the difference in osmotic pressure existing between the cell contents and the outside medium has not been demonstrated either for *Amoeba mira* or *Amoeba lacerata*, but if we include consideration of the osmotic pressure of the vacuoles, then the difference is appreciable but variable, being higher inside than outside.

Factors determining the rate of vacuolar output

In a given protozoan, the rate of fluid output depends on a number of factors. These factors and how they operate are not clearly defined but the following undoubtedly are involved:

(1) Respiration. The work of Kitching has demonstrated this. If the basic osmotic pressure of a cell is in equilibrium with that of the environment, the oxidative (aerobic or anaerobic) breakdown of foodstuffs will result in an increase in the osmotic activity of the cell as a whole even though these by-products are probably isolated into the vacuoles immediately by the formation of a precipitation membrane. It is readily seen from the facts presented here and by Mast and Hopkins (1941) that the rate of fluid elimination would depend upon the respiratory rate. Condensation of the by-products in the form of condensation granules would decrease their osmotic activity. While the rate of oxidation does have this controlling influence upon the rate of fluid elimination, the shape of the curve of Figure 5 is not determined by variations in oxidative rate. The oxygen supply, of course, was not absolutely constant but reasonably so. Since the curve represents an average, the variations are accounted for statistically.

(2) Food. In protozoa, food taken into food vacuoles and digested there adds to the over all osmotic activity of the cell, but if digested food is rendered insoluble by condensation into crystals or other food reserve bodies, this osmotic increase is nullified. This storage may not take place before appreciable increase in intake of

water has been affected. Also, the water engulfed with food would naturally have an influence on the rate of fluid uptake, but not upon the output as suggested by Kitching (1939).

(3) Agents causing polymerization or breakdown of substances in the protoplasm.

(4) Changes in osmotic activity of outer medium will cause increases or decreases in water intake and consequently the output. The permanence of these effects will depend upon the permeability of the membrane to the substances in the external medium.

Two vacuole systems as opposed to one

Amoeba mira (Hopkins, 1938) forms only one system of vacuoles. By this statement it is meant that all vacuoles which arise in this amoeba coalesce to form large cloacal vacuoles, which are eliminated in order of formation. The functions of excertion and digestion are accomplished by this system. On the other hand, *Amoeba lacerata* forms two entirely different sets of vacuoles: one which goes to make up the contractile vacuoles, and is excretory in function while the other system goes to combine with the engulfed food and is digestive in function. All protozoa probably can be separated into two groups: one primitive group, having only one vacuolar system; and a specialized group, having two systems.

SUMMARY

1. *Amoeba lacerata,* a fresh water amoeba, is able to adjust and live in any concentration of the salts of sea water up to 125 per cent.

2. Contractile vacuoles are formed in all concentrations and are separate from food vacuoles.

3. Food is engulfed with little or no water and small protoplasmic vacuoles coalesce with it. When digestion is completed, the food residues are eliminated with little or no fluid.

4. The major part of fluid elimination occurs by way of the contractile vacuoles.

5. Contractile vacuoles grow in size by coalescence and osmotic swelling.

6. The rate of fluid elimination is affected by a number of factors, but under constant optimal conditions it varies inversely with the concentration of the medium. It is suggested that the rate is proportional to the rate of catabolism.

7. The osmotic pressure of the protoplasm varies in the same direction as that of the outside medium. The protoplasm of completely adjusted amoebae is very nearly equal to that of the medium, the osmotic difference being less than that of 5 per cent sea water (0.13 atmosphere).

8. Contractile vacuoles have been shown to contain osmotically active substances; they shrink in hypertonic and swell in hypotonic solutions regardless of whether they remain in the cell or have been removed from the cell.

9. The volume of the amoeba is only temporarily dependent on the concentration of the medium. After adjustment, the volume shows no dependence upon concentration of the medium. If the amoeba is placed in a hypertonic solution the cell shrinks and then swells to its original volume within three hours time. Conversely, if placed in a hypotonic solution, it swells and then shrinks to its original volume.

10. The cell membrane is either permeable to substances in the medium especially when adjusting to a change in the concentration of the medium, or is capable of modifying its internal osmotic activity in some other way.

11. The contractile vacuoles are obviously excretory and also osmoregulatory organelles since they remove waste substances which would otherwise cause increases in the osmotic pressure in the cell.

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DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF ARBACIA PUNCTULATA. II. EFFECT OF THE MEDIUM ON RESPIRATION

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INTRODUCTION

The first section (Hayashi, 1945) of this investigation presented evidence for the existence of a factor in the seminal fluid of Arbacia which influences the duration of fertilizing capacity of the sperm of the same species. The experimental results indicated that the factor was adsorbed on the sperm surface, and subsequently was lost into the surrounding medium. Since Gray (1928a) has shown a relation between "mechanical crowding" of sperm and the length of metabolic life of sperm, the question at once arose as to whether the seminal fluid factor influenced the fertilizing power of sperm through an effect on the metabolism of sperm. Consequently, a series of experiments was done investigating the effects of seminal fluid on the respiratory activity of sperm.

MATERIALS AND METHODS

The microrespirometer used for these experiments was a compensating type, a modification of Krogh's respirometer.[1] It consisted of two similar vessels of conventional design connected through a common U-shaped manometer. To remove the carbon dioxide, filter paper wet with 20 per cent KOH was placed in the manometer vessels out of contact with the respiring cells. The manometer vessels were immersed in a constant-temperature bath maintained steadily at 25° C. The shaker, upon which the microrespirometers were mounted, moved to and fro in a straight line a distance of 15 centimeters. A steady rate of 40 cycles per minute thoroughly agitated the respiring sperm suspensions. The sensitivity of the respirometer was such that a respiratory rate of one mm.^{3} of oxygen consumed per hour could be detected in ten-minute readings.

Two points of difficulty were encountered in the course of these experiments. First, capillary action prevented the easy transfer of any liquids from the side-arm into the respirometer vessel. Second, the effect of dilution on the respiration of sperm was found to be very rapid, and therefore difficult to measure. To overcome these difficulties, a measured amount of packed sperm was placed on the glass wall of the manometer vessel above the point reached by a given amount of liquid in the vessel, when this liquid was agitated by the movements of the shaker. The packed sperm remained in place by their own adhesive action. When the time came to dilute the sperm in the medium to be studied, the adhered sperm on the side of the vessel were washed down into the liquid by a slightly greater agitation of the manometer vessel.

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[1] Kindly lent by Dr. Titus C. Evans, formerly of Iowa State University.

This technique overcame the first of the above-mentioned difficulties satisfactorily, but only partly the second. To wash down and to disperse completely the sperm cells in the medium required time in the order of minutes. Since, during this period, changing numbers of cells were being affected by the medium, neither the total respiration nor the total respiratory rate could be accurately measured. This effect was minimized by manipulation of the vessels and by extrapolation of the respiration curve.

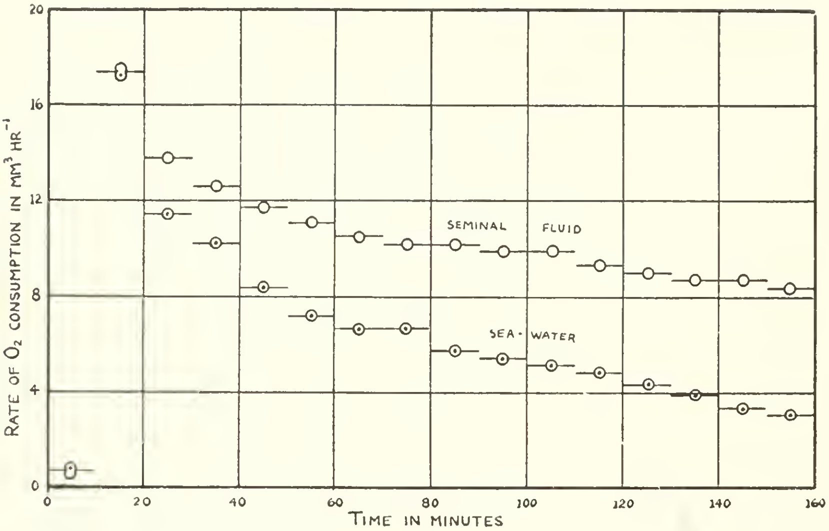

Figure 1. Effect of seminal fluid, sea water on respiratory rate of sperm.

The pH of the medium in the experiments here presented was not checked after each run, although it was known before the run, and has been published elsewhere (Hayashi, 1945). In later, similar experiments (to be published) the pH was adjusted to equality and checked before and after each run. In the time of the run, no change in pH occurred, and the results did not differ from those presented.

It was noted that the sperm in all the respiration experiments, irrespective of the medium used, and in the concentrations of these experiments, were dead at the end of five hours. A sharp rise in the rate of oxygen consumption occurs at about this time, due, probably, to disintegration of the sperm cells caused by the constant shaking of the manometers. This same effect was noted by Gray (1928b, p. 350). This effect afforded no trouble in the interpretation of the results, however, for it occurred uniformly in all sperm suspensions, and the pertinent data were obtained before the end of the five-hour period.

EXPERIMENTS AND RESULTS

For the first experiments, the respiration of sperm in seminal fluid was compared with the respiration of an equal amount of sperm in sea water. Each suspension contained 0.00155 cc. of packed sperm per cc. of medium. The contrasting changes in the respiratory rates of the two suspensions are shown in Figure 1. Using the same data, the total oxygen consumed was plotted as a function of time, to produce the curves of Figure 2.

The experiment showed that the seminal fluid did not affect the sharp increase in respiratory activity immediately following the dilution of the sperm. The main effect of the seminal fluid was to regulate, or delay, the sharp drop in respiratory rate shown by the sperm in sea water. Figure 1 also showed that the total amount of energy expended by equal amounts of sperm in the same length of time was greater when the sperm were suspended in seminal fluid.

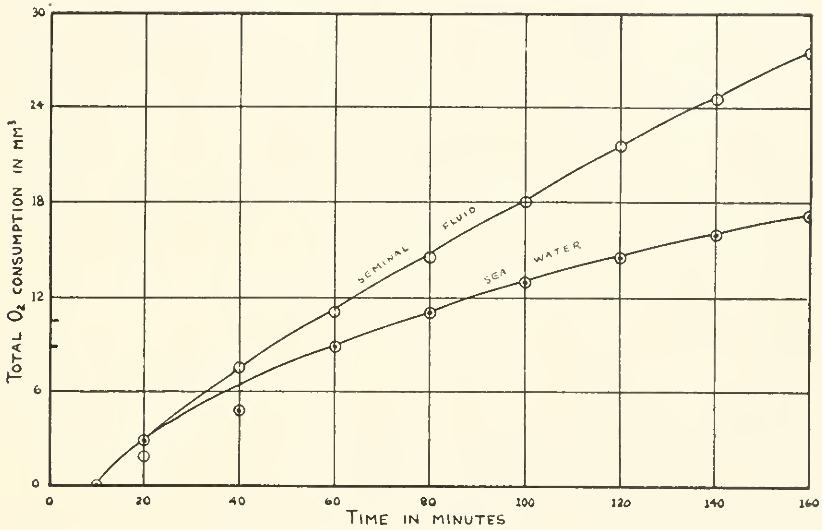

FIGURE 2. Total oxygen consumption as a function of time for sperm in seminal fluid and sea water.

As a further check on the effect of dilution on the respiration of sperm, one of Gray's (1928a) experiments was repeated, with both seminal fluid and sea water. A measured quantity of packed sperm (0.00155 cc.) was placed on the wall of the respiration chamber, and 1.0 cc. of medium was placed on the bottom of the vessel. In addition 0.5 cc. of medium was placed in the side arm, and this amount of medium was "dumped" over into the respiration chamber at an appropriate time. The results of the experiment are shown in Figure 3.

The results confirmed Gray, but compared with his results, the degree of rise in the respiratory rate at the second dilution was less. When the sperm concentrations used by Gray were compared with those used here, it was seen that the present sperm suspensions were far more dilute. Therefore, the results were taken to mean that the concentration of sperm in this experiment was near the upper limit to obtain the maximum burst of energy at first dilution from the amount of sperm used. In other words, greater original dilution of the packed sperm would not result in very much more initial activity.

This interpretation was partly confirmed by the following experiment. Lillie (1913) had found that egg water stimulated sperm to greater activity, and Gray (1928c) concluded from his experiments that egg water stimulated sperm to greater respiratory activity. However, Gray had used egg water as the first and only diluent for the sperm. In the following experiment, by contrast, the egg water was used as a diluent after the initial burst of activity induced by the original dilution of the medium.

The same procedure as the preceding experiment was employed. Instead of the respective media in the side arms, however, egg water was used, and this was "dumped" in at the appropriate time. The results are given in Figure 4. Study of Figure 4 showed that, after the original burst of activity, egg water, far from stimulating the sperm, seemed to inhibit them. The effect was probably due to the agglutination of the sperm, and seemed to overcome the slight stimulation due to dilution shown in Figure 3.

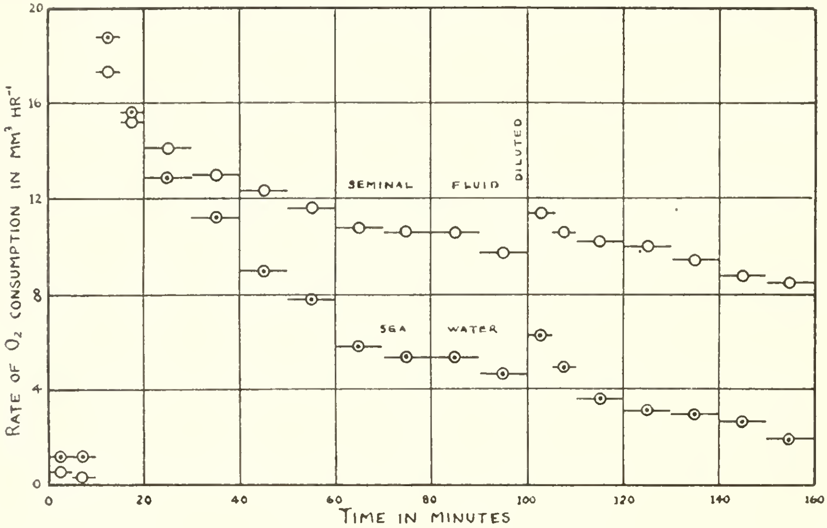

FIGURE 3. Effect of dilution by respective media on sperm in sea water and seminal fluid.

DISCUSSION

Analysis of the effect of the seminal fluid on sperm respiration

The respiratory rate of sperm in seminal fluid is compared with that of sperm in sea water in Figure 1. The seminal fluid does not seem to influence the initial steep rise in activity due to dilution. There was some variation shown in this first burst of respiration, but a check of all the runs made showed that the variations were not significant. In some cases the sperm in seminal fluid showed more intense activity at dilution; in others, the sperm in sea water were more active at dilution. The variations were caused probably by differences in the rate at which the sperm were washed from the sides of the respiratory vessels.

The effect of the seminal fluid is on the "senescent period" following the initial respiratory rise. The seminal fluid seems to prevent the rapid decline of the metabolic rate evident in the sea-water control. The effect of the seminal fluid on the respiration of sperm thus parallels the effect of seminal fluid on the fertilizing power of sperm. In both cases, the seminal fluid maintains the sperm at a high functional level for a longer time than does sea water. A comparison of the areas under the curves shows that sperm in seminal fluid expend more energy than an equal amount of sperm in sea water during the course of their active lives. There are two possible explanations for these results. It is possible that the sperm in seminal fluid utilized their internal store of fuel more completely than the sperm in sea water. The second possibility is that the seminal fluid is serving as a source of fuel for the sperm. A clue to these possibilities was sought by utilization of some of Gray's analytical methods.

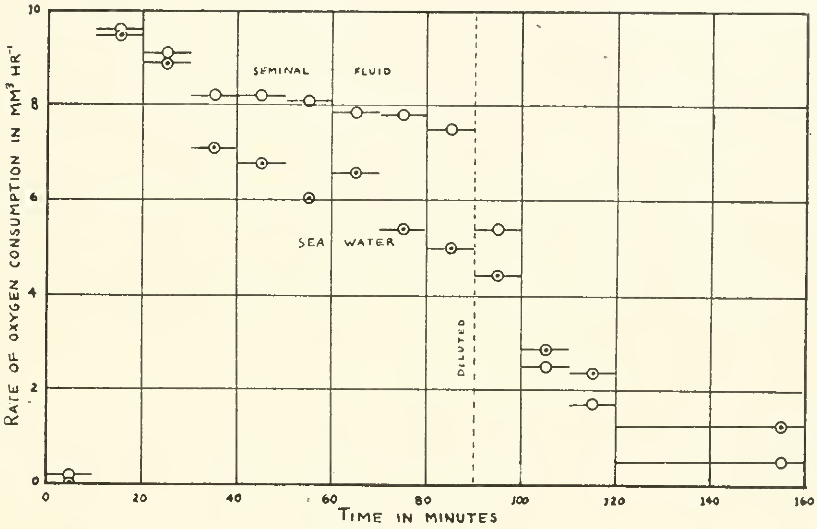

FIGURE 4. Effect of dilution by egg water on sperm in sea water and seminal fluid.

Gray (1928b) originally advanced the view that the sperm cell was a tiny combustion engine with a limited supply of fuel. From his original assumption, Gray derived the equation

$$\text{Activity} = \frac{dx}{dt} = k(a - x) \qquad \text{(Equation 1)}$$

which, integrated, gave

$$k = \frac{1}{t}\, ln_e \frac{a}{a - x}. \qquad \text{(Equation 1a)}$$

In this equation, x represented the amount of fuel used up in time, t; a, the initial, total amount of fuel in the sperm cell; and k, the activity produced by each unit of fuel consumed. Gray found that this "law of exponential decay of a homogeneous population" did not fit the experimental results. He, therefore, expanded the equation with a consideration of a heterogeneous population, and derived, as one form of the equation,

$$x = O_2\left(\frac{t}{\alpha + t}\right), \qquad \text{(Equation 2)}$$

in which O_2 represented the total oxygen consumed during active life; α, another constant; and x and t, possessing the same meanings as before. Gray found that this equation could be made to fit the experimental results, and concluded that the sperm population was heterogeneous in a special way, and that its activity decreased because of the depletion of an internal supply of energy. The notion of heterogeneity was used as a basis for a later, more detailed mathematical analysis (Gray, 1930).

The above methods were applied to the results of a number of experiments of this investigation. At two different values of time t in a single run, experimental values of the total oxygen consumption were arbitrarily selected, and the values of the constants k, a, O_2, and α in equations 1a and 2 were calculated. The values of the constants thus derived were then substituted back in the equations and the theoretical values of x for the entire run calculated and compared to the experimental values. The results showed that, with the proper selection of points, both equations 1a and 2 could be made to fit the experimental data rather closely, but other points could be selected to demonstrate that neither of the equations fit the experimental data. The latter case, in a typical experiment, is shown in Table I.

TABLE I

Comparison of the experimental and theoretical values of the total oxygen consumption of sperm in sea water and seminal fluid. Theoretical values calculated from equation 1a and equation 2

Time (minutes)	Total oxygen consumed in mm.^{3}					
	Sea water			Seminal fluid		
	Exp.	$k = .01275$ $a = 12.24$	$O_2 = 15.25$ $\alpha = 71.15$	Exp.	$k = .00876$ $a = 16.31$	$O_2 = 22.54$ $\alpha = 135.7$
0	0.00	0.00	0.00	0.00	0.00	0.00
10	1.05	1.45	1.87	0.95	1.36	1.55
20	2.07	2.75	3.32	1.99	2.62	2.90
30	3.09	3.89	4.50	3.08	3.77	4.08
40	4.06	4.89	5.46	4.06	4.83	5.13
50	4.96	5.77	6.26	5.06	5.78	6.07
60	5.84	6.55	6.94	6.04	6.66	6.91
70	6.68	7.23	7.53	6.97	7.47	7.67
90	8.08	8.36	8.49	8.60	8.90	8.99
100	8.70	8.86	8.88	9.37	9.52	9.56
110	9.23	(9.23)	(9.23)	10.09	(10.09)	(10.09)
210	11.41	11.40	11.37	13.80	13.72	13.69
220	11.50	(11.50)	(11.50)	13.94	(13.94)	(13.94)
255	11.76	11.76	11.90	14.31	14.56	14.71

It may be noted that the points selected are those covering the end of the run, and the data of this portion fit the experimental values rather closely, whereas wide variation is evident in the early portions of the run. Points selected near the beginning of the run, in contrast, would show a fit in that portion of the run, and variation at the end. Intermediate points, of course, would show an intermediate fit.

From such analyses, these conclusions may be drawn. (1) There is no need to assume heterogeneity of the sperm population whether in sea water or seminal fluid. (2) The simple "law of exponential decay" is not adequate to explain sperm activity; the decrease in activity of the sperm cells with time is not due to the exhaustion of an internal supply of fuel alone, whether the sperm cells are in sea water or seminal fluid.

Since sperm cells in sea water show a decline in activity whose controlling factor is not the exhaustion of their internal source of fuel, it is possible that even at the end of active life in this medium, the sperm cells contain unused, potential energy. The utilization of this unused energy by the sperm in seminal fluid would explain the increased oxygen consumption of the sperm cells in seminal fluid. There is, however, the possibility that the sperm in sea water reach the end of active life because of starving *plus* other factors. The question of nutrition by seminal fluid, therefore, is unsettled by the above analyses, although strong possibilities are afforded.

Gray (1928b) analyzed the senescence of spermatozoa from still other considerations. He assumed that the cause of the death of the spermatozoon was the accumulation of products of metabolic activity inside the sperm cell. This would render inactive part of the active system originally present in the cell.

$$\text{Active system} + \text{product of activity} \rightleftarrows \text{inactive system.}$$

Based on this concept Gray derived an equation which he called the "theory of autointoxication."

$$x = \sqrt{\frac{2Ka}{b}}\,\sqrt{t}. \qquad \text{(Equation 3)}$$

Here x, a, and t designated the same quantities as before, and K and b were constants. Equation 3, based primarily on the assumption of a homogeneous population, showed that the amount of fuel, x, consumed in time t was directly proportional to the square root of t. Therefore, the total oxygen consumed should show a straight-line relationship to the square root of the age of the active suspension. Gray found that this relationship did not hold for sperm in sea water, for toward the end of active life the points fell below the expected values. He concluded that part of the active system was not only being inactivated, but also being irreversibly destroyed.

Equation 3 was applied to the experimental figures of the present investigation. When the total oxygen consumption of the sperm in sea water is plotted against the square root of the time, the results confirm Gray's findings, for, toward the end of the run, the points fall below the expected values. (The variation from the straight-line relationship at the beginning of the run is due to the time requird for the cells to be completely dispersed in the medium.) For the sperm in sea water, therefore, an irreversible destruction of part of the active metabolic system is taking place toward the end of the run (Fig. 5). In contrast, the straight-line relationship is followed for a much longer time in seminal fluid in an equivalent run (Fig. 6). In some of the experiments, a departure from the straight-line relationship occurred in the seminal fluid, but in all cases, this departure occurred very much later from the beginning of active life. In the seminal fluid, therefore, the irreversible destruction of the active system is much delayed, as compared to the case of the sperm in sea water. The decay of activity of the sperm in seminal fluid is apparently due, at least for a great part, to a process of autointoxication.

The difference between the metabolism of sperm in sea water and in seminal fluid apparently lies in the fact that some sort of metabolic system is kept intact for a longer time when the sperm cells are suspended in seminal fluid. The sperm and a factor in the seminal fluid, therefore, constitute a closed system. In sea water, part of this closed system is destroyed quite early during the active life of the sperm.

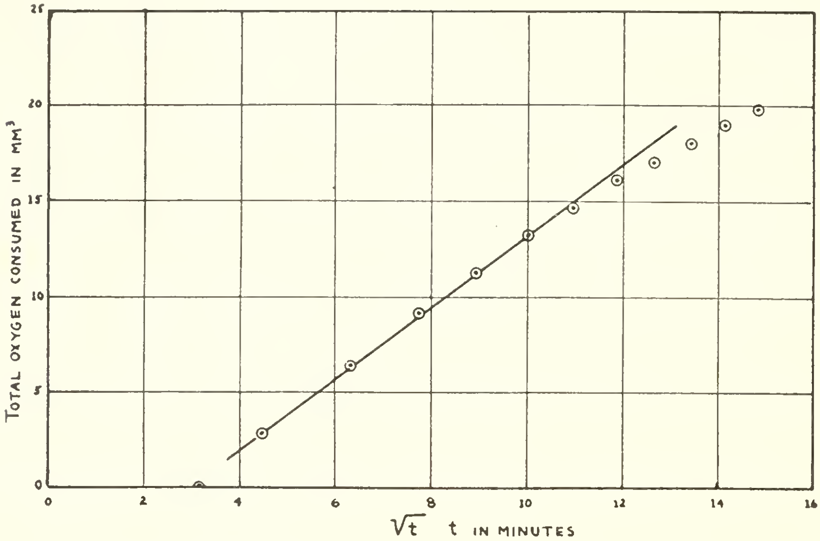

FIGURE 5. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in sea water.

The above findings, supported by the knowledge that seminal fluid contains no reducing sugars (Hayashi, 1945), lends further support to the idea that the effect of seminal fluid on sperm respiration is not due to the replenishment of a store of energy, but rather to the maintenance of a metabolic system. Other experiments (unpublished data) measuring the R.Q. of sperm cells both in sea water and seminal fluid show a characteristic carbohydrate metabolism for these cells, further supporting the contention that the seminal fluid does not provide added nutrients for the sperm cells. It may be concluded that, although there is the possibility that sperm can be nourished experimentally, it is most important for future experimentation that the system, of which the sperm cells are only a part, be kept intact. Workers who have diluted sperm cells in sea water have diluted not only cells, but, also, system.

Both the fertilizing power (Hayashi, 1945) and the respiratory activity of sperm are maintained by a common factor, the seminal fluid. As a first possibility, the role of the seminal fluid factor may be considered fundamental to the respiratory activity only, the fall in fertilizing power being a manifestation of the loss of activity of the sperm cells. In other words, the fall in fertilizing power is only a secondary effect in relation to the seminal fluid factor. The second possibility is that the seminal fluid plays a part directly in both fertilization and respiration. Lack of data does not permit the choice of these possibilities. However, the fact that a seminal fluid factor influences sperm respiration is implicit in both possibilities.

A mechanism, tentatively proposed earlier as the wearing off of a protein substance from the surface of the sperm cell, was adequate to explain the loss of fertilizing power of sperm in sea water. The same mechanism is also applicable to the results of respiration studies, if the assumption be made, that the removal of each molecule of protein substance from the surface of the sperm cell releases to the sperm cell a finite amount of the total internal energy. The rate of sperm respiration thus would be controlled by the rate of removal of substance from the sperm surface, which is in turn controlled by other factors. The principal factor affecting the rate of removal of a sperm-surface-substance is the "mechanical crowding" factor studied by Gray (1928a), who found that sperm cells exhibited a burst of activity when diluted. This was confirmed in the present study (Figs. 1, 3, 4). According to the mechanism proposed, the rate of loss of sperm-surface-substance is inhibited when the cells are in a crowded condition, but when relatively far apart, the removal of surface-substance is enhanced.

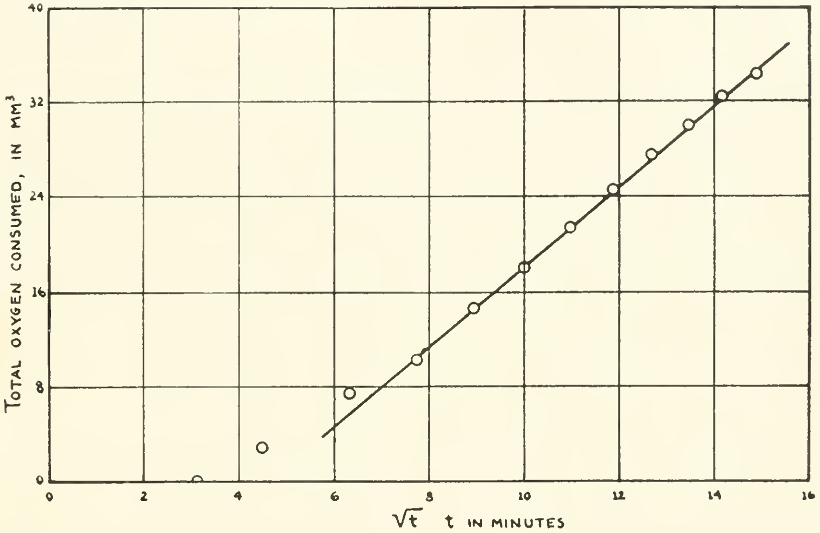

FIGURE 6. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in seminal fluid.

The removal of sperm substance from the cell surface presumably constitutes the irreversible destruction of part of the active system noted in Fig. 5. In seminal fluid, however, the replacement of the surface-substance would delay the onset of this irreversible destruction (Fig. 6), the senescence of the sperm cells being conditioned by internal autointoxication, and perhaps also by depletion of the internal energy. It would seem that for the sperm cells in sea water, the destruction of part of the system, in addition to autointoxication and probable depletion of fuel, controls the fall in activity.

It is understood that the mechanism as outlined is tentative at best, but it serves as an explanation of the facts so far known. The facts fit well with the earlier results of the effect of seminal fluid on the fertilizing capacity of sperm. It may be concluded that possibly a common mechanism underlies the effect of seminal fluid on the fertilizing function and the respiration of sperm. The relationship between the surface-substance and the respiratory mechanism of the sperm cell was not investigated. The action of the surface-substance while attached to the sperm cell is possibly enzymatic.

Effect of egg water on sperm respiration

Egg water does not stimulate the respiration of sperm cells but seems to condition a sharp drop in the respiratory rate (Fig. 4). When egg water was added to sperm suspended in sea water and, also, to sperm suspended in seminal fluid, the effect was a sharp decrease in the respiratory rate. This decrease of metabolic activity upon dilution with egg water is in contrast to the effect of dilution with sea water and seminal fluid shown in Figure 3. The increase in respiratory rate brought about by dilution with sea water and seminal fluid shows that even at the dilution used, "mechanical crowding" was still apparently a factor inhibiting the respiration of spermatozoa. Dilution with egg water also relieved the "mechanical crowding," but the agglutinating effect of egg water apparently overcame the effect of dilution so that the respiratory activity decreased.

The stimulation of metabolism by egg water noted by Gray (1928c) and Carter (1930) is not confirmed in these experiments. However, it may be recalled that Carter (1931) had found no stimulation of ripe sperm by egg water. In addition, the egg water of the present investigation was added, not to undiluted sperm, but to sperm that had already been activated by an initial dilution. Therefore, it is possible either that egg water did not affect the respiration of sperm at all, or that the difference in the time of addition of egg water was the cause of the disparity of the results of Gray's experiments and the results of the present investigation.

SUMMARY

1. Seminal fluid has the property of delaying the fall of respiratory activity of the sperm after the original burst of activity upon dilution.

2. Gray's "theory of exponential decay" is not adequate to explain sperm metabolic activity, whereas the "theory of autointoxication" fits the activity of sperm cells suspended in seminal fluid.

3. The seminal fluid delays appreciably the onset of an irreversible destruction of part of the metabolic system.

4. Fertilization studies have led to the formulation of a tentative mechanism based on the adsorption of a protein substance and its removal from the surface of the sperm cell.

5. The proposed mechanism also explains adequately the results of the respiration experiments. Therefore, it is concluded that a seminal fluid factor, by its action while on the surface of the sperm, influences both the fertilizing capacity and the respiratory rate of spermatozoa.

6. Egg water added to a sperm suspension after the original dilution, causes a sharp decrease in the respiratory rate.

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THE EFFECTS OF LITHIUM CHLORIDE ON THE FERTILIZED EGGS OF NEREIS LIMBATA

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INTRODUCTION

The action of the lithium ion on the eggs of certain animals has long been known. Using a number of lithium salts, Herbst (1892) obtained abnormal embryos of two types. In the first group, the endoderm was found not inside the body, but outside; these forms are designated "exogastrulae." Larvae of the second group showed an apparent conversion of ectoderm into endoderm so that in extreme cases, the whole blastula wall was endodermized (Herbst, 1892, 1893, 1943). Herbst concluded that this was a specific and typical affect of lithium, a view shared by Gurwitsch (1895), Morgan (1902), and other early investigators.

Studying the effects of 0.2 per cent to 1.0 per cent solutions of lithium chloride on frog and toad eggs, Gurwitsch found that in the resulting embryos, gastrulation was abnormal, but no exogastrulae were produced. Morgan (1902), using similar concentrations of lithium, obtained embryos in which the black hemisphere had sunk into the interior of the egg, but here again there was no definite indication of exogastrulation. Experiments by Holtfreter (1931) showed that amphibian exogastrulae could be obtained if the blastulae were treated with a modified Ringer solution. Further experiments to ascertain the effects of lithium on the amphibian egg were carried out by Bellamy (1919) who observed a number of cases of fusion of the lateral sense organs of the larvae, as well as abnormalities in gastrulation. Töndury (1938) treated urodele embryos in early stages of gastrulation with LiCl and described a high percentage of head and foregut abnormalities in the larvae. Still another effect of lithium on urodele eggs was observed by Cohen (1938) who treated early gastrulae or late blastulae and observed cases in which the myotomes formed a continuous sheet across the midline, separating the nerve cord and notochord. He also obtained embryos in which exogastrulation had occurred, seemingly as a result of the treatment with lithium.

The effects of LiCl on the eggs of the pond snail, *Limnaea stagnalis*, were studied by Raven (1942) who was able to produce forms which he designates as exogastrulae. He also describes a number of larvae in which varying abnormalities of the eyes were apparent. These experiments are of interest in connection with the present series because the egg of Limnaea, like that of Nereis, is an example of the so-called "mosaic" type of development.

A wide variety of chemical and physical agents has since been found to produce echinoderm exogastrulae, thus invalidating the theory of an ion specificity. These agents include hypotonic sea water, isotonic solutions of magnesium chloride and lithium chloride, and combinations of isotonic solutions of magnesium chloride, sodium chloride, potassium chloride, and calcium chloride as studied by Waterman (1932); solutions of nickel chloride and copper chloride (Waterman, 1937); lithium in low concentrations augmented by carbon monoxide (Runnström, 1935); and 97 per cent carbon monoxide with 3 per cent oxygen in the presence of light (Runnström, 1928a). MacArthur (1924) treated sea urchin eggs with calcium chloride, stale or diluted sea water, copper sulfate, mercuric chloride and potassium cyanide, and obtained exogastrulae. Using "auxin," glycogen and potassium chlorate, Motomura (1934) likewise obtained echinoderm exogastrulae.

Most of the eggs previously tested with lithium have been those of echinoderms and amphibians, both of which are characterized by an invaginative type of gastrulation. It seemed, therefore, that an egg showing a strictly epibolic form of gastrulation should be tested, in an effort to ascertain if the same effect could be produced. The following experiments were performed to study the effects of lithium on the fertilized eggs of the annelid, *Nereis limbata*.[1]

METHODS

Gametes of the heteronereis form of *Nereis limbata* were obtained at Woods Hole from June to September, during appropriate phases of the moon. Usually the eggs from one female were sufficient for an average experimental series; they were inseminated according to the methods outlined by Just (1939) and were then washed with freshly drawn, filtered sea water.

The fertilized eggs were allowed to remain undisturbed for a period of 75 minutes after insemination; at the end of this time (shortly before the first cleavage), they were transferred with as little sea water as possible to a series of 25 cc. stender dishes, to which the various solutions of LiCl were then added. Appropriate controls were kept in all series, these being cultured in filtered aerated sea water which was changed at daily intervals. A stock solution of 0.54 M LiCl in distilled water was used for all experimental mixtures; such a solution is approximately isotonic in all dilutions with sea water. Experimental mixtures were designated according to the percentage of this stock solution used—e.g., a "15 per cent solution" indicates that 85 cc. of filtered aerated sea water were added to 15 cc. of the 0.54 M LiCl stock solution. About 15 cc. of the mixture were added to each stender dish; at intervals ranging from 15 minutes to 36 hours, the solutions were decanted carefully and the eggs washed in three or four changes of sea water. LiCl-sea water mixtures ranging from 2 per cent to 100 per cent were tested for varying periods of time. No attempt was made to control the room temperature, which varied from 20° C. to 28° C. However, all cultures were kept in moist chambers which stood in running sea water; the temperature of this sea water averaged about 20° C., and did not vary more than one or two degrees at any time during the entire series of experiments.

Less extensive test series were also conducted for purposes of comparison, using the eggs of Nereis from which the vitelline membrane had been removed, according to the method described by Costello (1945a).

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[1] The author wishes to express sincere appreciation to Prof. D. P. Costello for advice and encouragement throughout the course of the investigation, to Prof. Viktor Hamburger and Dr. Charles Packard for use of laboratory space at the Marine Biological Laboratory during the summers of 1944 and 1945, to Prof. B. H. Willier for his kindly interest, and to Mr. John S. Spurbeck for valuable assistance with the drawings.

RESULTS

The most striking characteristic of the experimental larvae is the complete absence of exogastrulation, or of any definite evidence of true vegetalization. Abnormal larvae occurred to some extent in almost all the cultures; these abnormalities included the absence of an apical tuft, lengthening of the prototrochal cilia (Fig. 2A), absence of these cilia in varying degrees (Fig. 2), absence or abnormality of the eye spots (Fig. 3), deficiencies in the number of prototrochal cells (Fig. 3), atypical seta sacs with derangements in the number and position of these organs in older larvae (Fig. 4), and abnormalities in the number and position of the oil droplets (Figs. 2C, 3D, 4B). Most of the experimental larvae had abnormalities of the anal and prototrochal pigments; these abnormalities included both the absence of pigment and an abnormal concentration of pigment granules in various regions of the larvae (Figs. 2, 3, 4, 5). The specific occurrence of these anomalies was roughly proportional to the severity of treatment, being far more marked in cultures which were exposed to higher concentrations for sublethal periods. No evidence of twinning was observed.

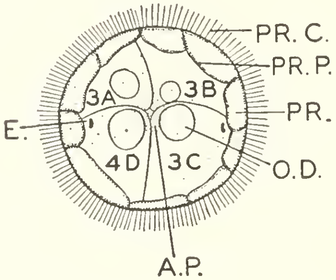

FIGURE 1. A normal Nereis trochophore at about 30 hours; polar view. Pr. C. = Prototrochal cilia; Pr. P. = Prototrochal pigment; Pr. = Prototrochal cell; O. D. = Oil droplet; A. P. = Anal pigment; E. = Eye spot; 3A, 3B, 3C, 4D = Entomeres. This and all subsequent text-figures are semi-diagrammatic, based on camera lucida drawings of the living larvae.

Complete data describing the results of treatment with the varying concentrations for varying periods are contained in Table I. It is evident that concentrations of 2 per cent to 4 per cent acting for periods up to 12 hours produce larvae which are almost completely normal; similar trochophores result when 5 per cent to 10 per cent solutions are applied for one to 5 hours. However, when allowed to act for periods of 5 to 24 hours, the same concentrations affect the eggs so that the resulting larvae show marked deficiencies in the number of prototrochal cells, and are usually completely devoid of the normal anal and prototrochal pigment. Abnormalities such as these occur quite commonly in all series; some extreme examples are shown in Figure 2. For purposes of comparison, a normal trochophore is shown in Figure 1. Accurate quantitative observations of the exact deficiency in the number of prototrochal cells were impossible in most cases.

Twelve per cent solutions acting for periods of 1½ to 5 hours result in trochophores which are essentially normal, but if allowed to remain on the eggs for 5 to 24 hours, bring about pigment and prototrochal anomalies of the type described above.

TABLE I

Effects of lithium treatment of Nereis eggs

Percentage[2]	Duration of treatment in hours	Appearance of 26–30 hour larvae
2%	1½	Normal
	5	Normal
	8	Mostly normal; some with abnormalities in number and position of oil droplets
	23	Mostly normal; some with abnormalities in number and position of oil droplets
3	8	Many with incomplete prototroch and with abnormalities in number and position of oil droplets
	10	Many with incomplete prototroch and with abnormalities in number and position of oil droplets
	24	Abnormal distribution of oil droplets, otherwise normal
4	1½	Normal
	5	Mostly normal
	8	Mostly normal
	12	Normal in form; somewhat sluggish in movements
5	14	Normal
	16	Mostly normal
	18	Mostly normal
	20	Some prototrochal and pigment abnormalities
	22	Pigment and prototrochal abnormalities
	26	Considerable variation in amount of pigment present
	28	Variations in amount of pigment present
	30	More marked pigment abnormalities than after 28-hour treatment
	32	Pigment and prototrochal abnormalities
	36	Dead
6	1½	Normal
	5	Normal, but sluggish in movements
	8	Mostly normal
	12	Normal, but sluggish in movements
	23	Quite abnormal; prototrochal and pigment abnormalities
7	1	Normal
	2	A very few cases with oil droplet deficiencies
	5	Some cases with oil droplet deficiencies
	24	Prototrochal cell deficiencies; sluggish in movements; pigment often absent
8	1½	Normal
	5	Some cases with prototrochal and pigment abnormalities
	8	More marked pigment abnormalities
	12	Severe prototrochal deficiencies; no pigment
	23	Severe prototrochal deficiencies; no pigment. Oil droplets abnormal in number and position. Many larvae without apical tuft

[2] Percentages in the table refer to percentages of 0.54 M stock solution of lithium chloride with sea water: 2 per cent = 2 cc. 0.54 M LiCl + 98 cc. sea water.

TABLE I—*Continued*

Percentage[2]	Duration of treatment in hours	Appearance of 26–30 hour larvae
10	1	Some localized deposits of pigment; some pigment present in most cases
	4	Some pigmented, some unpigmented. A few cases of abnormality in amount and position of anal pigment
	8	Pigment abnormalities; prototrochal cells seem fairly complete
	12	Pigment abnormalities; prototroch fairly normal
	14	No pigment; marked defects in number of prototrochal cells
	16	No pigment; marked defects in number of prototrochal cells
	18	No pigment, no cilia, no apical tuft
	20	No pigment, no apical tuft. Abnormalities in number and position of oil droplets
	24	No pigment, no cilia, no apical tuft. Abnormalities in number and position of oil droplets
	26	No pigment; marked prototrochal deficiencies. No apical tuft; oil droplets very abnormal in number and position
	28	No pigment; prototrochal defects marked. Oil droplets abnormal in number and position
	36	Dead
12	1½	Fairly normal; some abnormalities in number of prototrochal cells and oil droplets
	5	Many deficiencies of pigment and prototroch; oil droplets abnormal in number and position
	8	Pigment deficiencies; prototroch and oil droplets abnormal in number and position
	12	Some abnormalities in distribution of oil droplets; no pigment. Prototrochal deficiencies; no apical tuft
	23	Dead
14	1½	Mostly normal; some pigment deficiencies
	5	No pigment; marked prototrochal deficiencies
	8	No pigment; prototrochal deficiencies; no apical tuft
	23	Dead
15	1	Mostly unpigmented; prototrochal deficiencies. Anal pigment present in many cases. Oil droplets abnormal in number and position
	2	Unpigmented; prototrochal deficiencies; anal pigment present in some cases. Oil droplet abnormalities
	4	More marked pigment defects; severe prototrochal deficiencies. Some few cases of endodermal extrusion
	6	Highest incidence of endodermal extrusion: 2%–7%
	8	Somewhat fewer cases of extrusion than in 6-hour culture
	10	Some few cases of endoderm extrusion
	12	A very few cases of endodermal extrusion
	14	No pigment; marked prototrochal defects. No apical tuft .
	16	No pigment; prototrochal defects; no apical tuft
	20	No cilia, no pigment, no apical tuft. Severe prototrochal deficiencies
	24	No pigment, no cilia, no apical tuft

TABLE I—*Continued*

Percentage²	Duration of treatment in hours	Appearance of 26–30 hour larvae
17	1	Anal pigment present; prototrochal pigment absent in many cases. Considerable number of oil droplet abnormalities
	2	No pigment; oil droplet abnormalities; prototrochal deficiencies; no apical tuft
	3	No pigment; oil droplet abnormalities in number and position. Prototrochal deficiencies, no apical tuft
	6	Marked oil droplet deficiencies and abnormalities in position; no pigment. No apical tuft; prototrochal cell deficiencies
	8	Many amorphous forms; no cilia; abnormalities in number and position of oil droplets
	10	No cilia, no pigment, no apical tuft
	14	Dead
20	1	Normal
	2	Pigment abnormalities; some localization of pigment
	4	No pigment; prototrochal cell deficiencies; apical tuft apparently missing; oil droplets abnormal in number and position
	8	Marked oil droplet deficiencies; severe prototrochal defects
	12	Oil droplet abnormalities; marked prototrochal deficiencies
	14	Dead
25	1	Prototrochal cell deficiencies; some pigment defects
	2	No pigment; prototrochal cell deficiencies; abnormalities in number and position of oil droplets
	4	Severe prototrochal deficiencies; no apical tuft. No anal pigment
	8	Prototrochal deficiencies; pigment abnormal. Oil droplet abnormalities in number and position
	12	Marked prototrochal cell deficiencies, no pigment, no apical tuft
	14	Dead
30	2	No pigment; marked prototrochal deficiencies. No apical tuft in most cases
	4	Dead
35	2	No pigment; marked prototrochal deficiencies. No apical tuft
40–90	1	Dead
50	½	No pigment; prototrochal deficiencies. Apical tuft usually absent
100	¼	Prototrochal and pigment deficiencies
	½	Dead

Treatment of the eggs with 15 per cent solutions for one to 4 hours produces pigment and prototrochal abnormalities, but after 6, 8, and 10 hours of treatment, the resulting larvae are marked by peculiar endodermal derangements which were at first thought to be the result of exogastrulation. However, closer examination of these cases revealed that the extruded cytoplasm was non-cellular insofar as could be determined, although it had the characteristic "glassy" appearance of endodermal cytoplasm; usually these larvae were marked by the complete absence of cilia (Fig. 5).

Frequently cases were observed in which the endoderm extended up under the surmounting cap of ectoderm. The cleavage pattern of the experimental eggs of this series showed no abnormality through the fourth cleavage. Repeated experiments with this treatment and with exposure of the eggs to concentrations of 12 per cent and 17 per cent for varying periods of time definitely established the fact that the abnormalities occur only after the treatment described above. It does not seem likely that such an extremely narrow range of dosage would be necessary for the production of true exogastrulae, inasmuch as these forms are produced in sea urchin eggs after a rather wide variety of treatments.

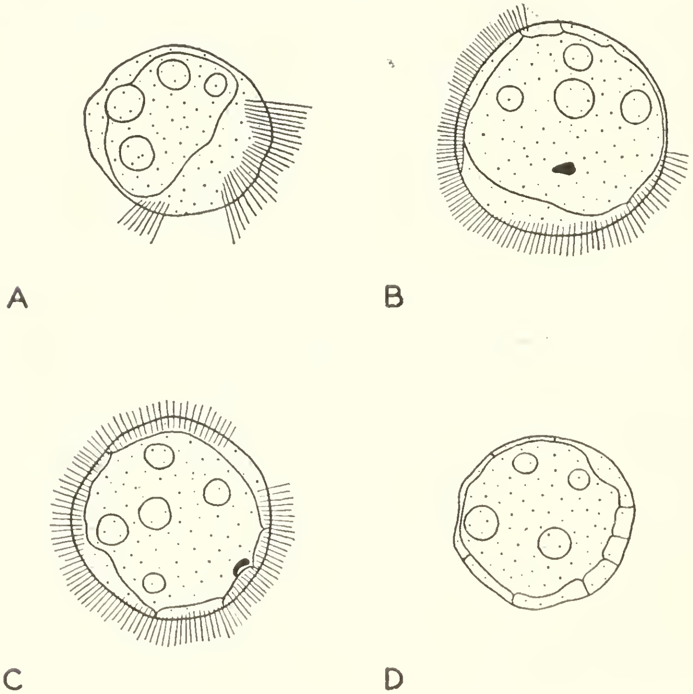

FIGURE 2. Typical 26–30 hour abnormal trochophores, showing anomalies in ciliary band, pigmentation and eye spots. Anal pigment and prototrochal pigment are absent in all cases; Figure 2A shows lengthening of the prototrochal cilia.

Exposure of the eggs to a 17 per cent solution resulted in generally abnormal trochophores after a treatment of one hour, and in more pronounced pigment and prototrochal abnormalities if treated for longer periods, up to the lethal point at 14 hours. No larvae with the endodermal derangements described above were noted in any of the cultures in this series. The effects of a 20 per cent solution for comparable periods of time approximate those described for a 17 per cent treatment; a concentration of 25 per cent produces pigment and prototrochal abnormalities after treatment for 1, 2, 4 and 8 hours. These defects also result from treatment for 2 hours with a 30 per cent solution. Concentrations of 40 per cent to 90 per cent are lethal after treatment for one hour, and a 100 per cent solution has the same effect after ½ hour; the 100 per cent concentration produces pigment and prototrochal defects if applied for 15 minutes, so that its range of dosage is apparently very narrow. The reduction of the prototroch seems to increase quantitatively with an increase in the concentration of lithium and the duration of application.

Denuded Nereis eggs proved to be extremely sensitive to the effects of lithium, even in low concentrations for short periods of time. In all series, the controls showed considerable abnormality in shape and in the distribution of pigment, so that it is difficult to come to any definite decision as to the specific effects brought about by the lithium. However, in the cases in which membrane removal was not complete, the larvae survived fairly well, and in none of these cases were any indications of exogastrulation observed. After treatment for 2 hours with 15 per cent LiCl, some of the completely denuded larvae seemed to show signs of exogastrulation, although no conclusions can be drawn from this because of the small number of cases. Treatment of the denuded eggs with 5 per cent solutions for periods longer than 4 hours killed the eggs in late stages of cleavage, and no definite effects could be noted in larvae surviving in cultures which had been exposed for periods of 1 and 2 hours.

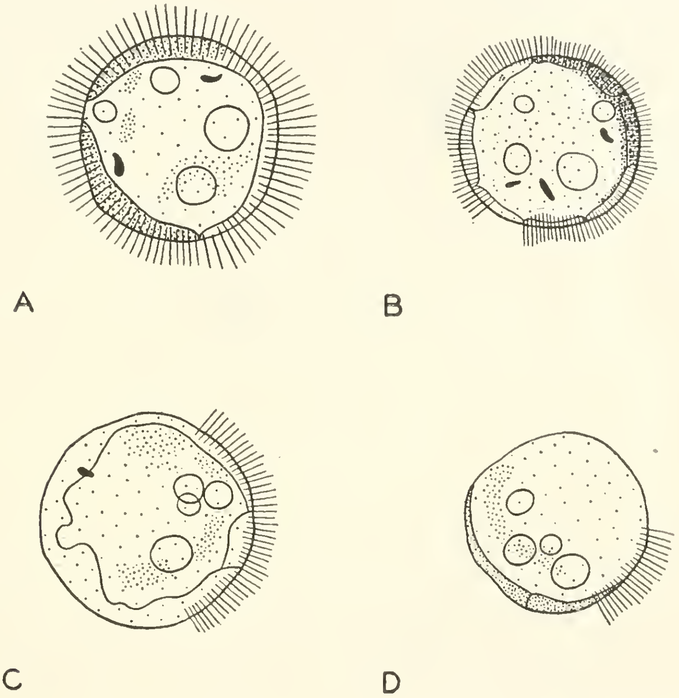

FIGURE 3. Abnormalities involving pigment, eye spots, cilia, and oil droplets after various treatments with lithium. Figure 3A is almost normal except for the heavy concentrations of anal and prototrochal pigment; Figure 3B indicates an abnormality in the number and position of the eye spots and in the size of these structures.

DISCUSSION

From the foregoing results, it is apparent that the main effect of the lithium is to produce a reduction in the number of prototrochal cells, and in the quantity of anal and prototrochal pigments, together with effects on the eye spots and cilia. The absence of the apical tuft in most cases of prolonged or concentrated treatment indicates that the la-ld cells may be affected directly or indirectly, since it is known from the observations of Wilson (1892) that this quartet is associated with the formation of the apical tuft. It is possible that the abnormalities observed in the seta sacs of older larvae (Fig. 4) may be due to the early action of the ion on the material destined to be incorporated into the 2d cell.

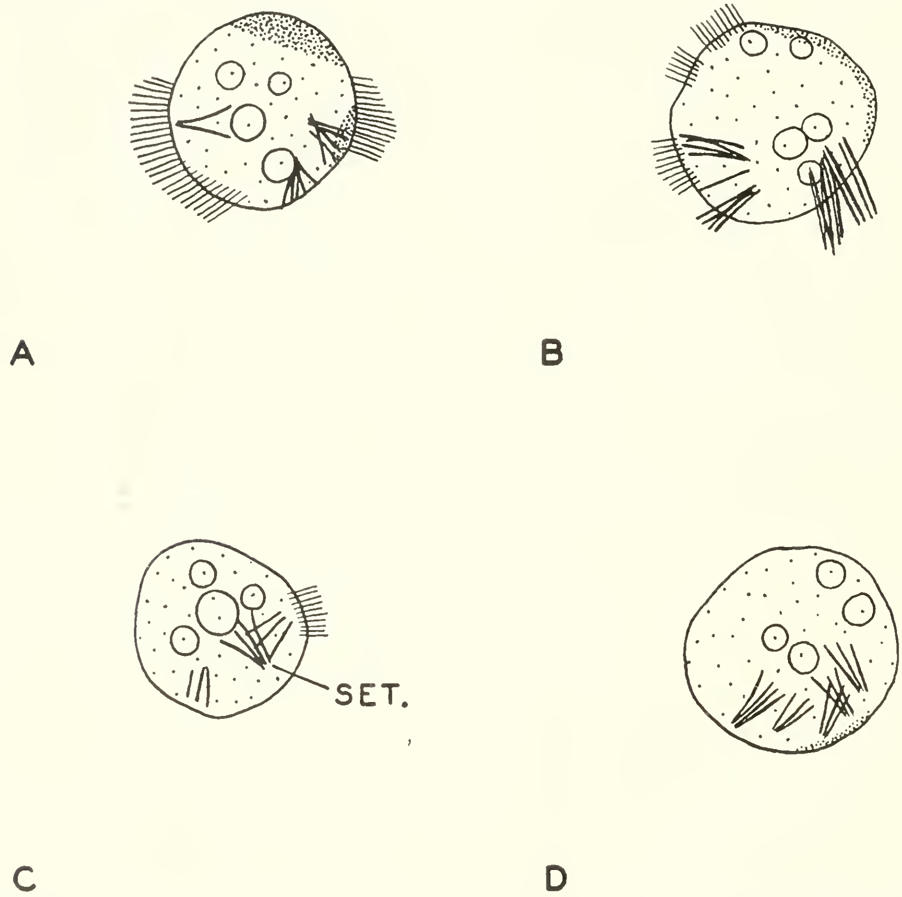

FIGURE 4. Marked abnormalities in setae of older larvae (ca. 53 hours). Figure 4B also shows an atypical number of oil droplets. Set. = Setae.

The absence of any true cases of exogastrulation in these experiments is quite striking. As was noted above, most of the forms in which this abnormality has been observed are marked by a type of gastrulation in which invagination plays a main role. In the amphibian, formation of the endoderm is accomplished by a complex series of cell movements, as a result of which a directed and organized migration of cells brings about invagination of most of the vegetal hemisphere at the region of the blastopore. Meanwhile, the animal cells grow down to cover the vegetal region, thus forming the ectoderm of the embryo. The yolk material itself is invaginated in these forms and comes to be enclosed by the endoderm. Gastrulation in the echinoderms is likewise thought by Herbst (1893) to be brought about by an invaginative process. The strictly epibolic form of gastrulation exhibited by the egg of Nereis, on the other hand, is accomplished by a downgrowth of the ectomeres, so that eventually they cover the four entomeres. Such a movement seems to be associated with the presence of a "cellular affinity" as postulated by Costello (1940, 1945a) in connection with his experiments on the development of isolated blastomeres of this egg. Since no exogastrulation occurred, it may be assumed that this dynamic association of ectomeres and entomeres is not radically disturbed. However, the abnormalities in the number and position of the oil droplets in the four entomeres indicate that the lithium may exert some effect on the process of cytoplasmic segregation (Costello, 1945b) preceding the formation of the 3A, 3B, 3C and 4D cells.

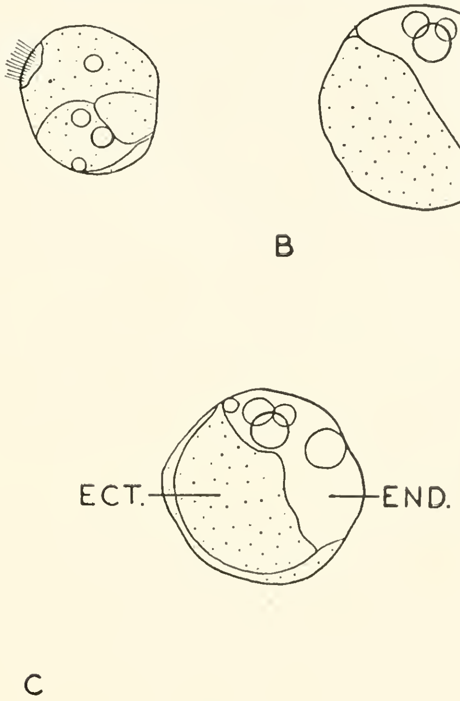

FIGURE 5. Abnormal trochophores resulting from treatment of eggs with 15 per cent LiCl for 6, 8 and 10 hours. Figure 5A shows no particular endodermal derangements, but has only one small tuft of cilia and is quite atypical in shape. Figures 5B and 5C show examples of "endodermal extrusions." Cell boundaries were not visible except as indicated. Ect. = Ectoderm; End. = Endoderm.

No distinct line of demarcation can be drawn between the direct and the indirect effects of lithium in these experiments, since the ion is brought into contact with the egg before cleavage has occurred, and, in the case of the longer exposures, may remain until considerably after the completion of gastrulation. Runnström (1928b), observing lithium-treated sea urchin eggs under dark-field illumination, presented evidence that the element actually penetrates the cells. Spek (1918) maintained that the action of the lithium ion was brought about through its production of a precipitation and swelling effect on the surface of the vegetative cells. Thus, the exact mode of action remains obscure, but it appears fairly clear in the case of the egg of Nereis that exogastrulation is not produced, at least not in cases where the vitelline membrane is present.

The effects produced on the pigment of the trochophores seem to be at random, since the anal pigment may occur without the presence of the prototrochal pigment, and vice versa—or both may be present in varying degrees. This action possibly is related to the orientation of the eggs with respect to the bottom of the dish, or to each other; although no particular effort was made to keep the eggs suspended in the solution, the jelly serves to support them during the early stages of development, thus allowing relatively free access of the lithium to all surfaces.

Summary

1. The fertilized eggs of *Nereis limbata* were treated with mixtures of sea water and a 0.54 M stock solution of LiCl, ranging from 2 per cent to 100 per cent for periods of 15 minutes to 36 hours. Treatment was begun 75 minutes after insemination of the eggs, shortly before the appearance of the first cleavage.

2. No exogastrulae were observed. There were a few cases of marked abnormalities in the endodermal components of the trochophores within a very narrow range of treatment (15 per cent for 6, 8 and 10 hours).

3. The main abnormalities observed in the experimental larvae were: Absence of the apical tuft, lengthening of the prototrochal cilia, absence of these cilia in varying degrees, abnormalities in the anal and prototrochal pigments, absence or abnormality of the eye spots, deficiencies in the number of prototrochal cells, atypical seta sacs, abnormalities in the number and position of oil droplets. The degree of abnormality in these cases seemed to be roughly proportional to the severity of treatment.

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE CHATTON AND LWOFF (ORDER HOLOTRICHA, SUBORDER THIGMOTRICHA). II. HYPOCOMIDES MYTILI CHATTON AND LWOFF, HYPOCOMIDES BOTULAE SP. NOV., HYPOCOMIDES PARVA SP. NOV., HYPOCOMIDES KELLIAE SP. NOV., AND INSIGNICOMA VENUSTA GEN. NOV., SP. NOV.

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INTRODUCTION

The genus *Hypocomides* was proposed by Chatton and Lwoff (1922a) to include two species: *Hypocomides mytili*, from *Mytilus edulis* L., and *H. modiolariae*, from *Modiolaria marmorata* (Forbes). These authors gave no formal diagnosis of the genus *Hypocomides*, however, and their incomplete descriptions of the two species are not supplemented by illustrations. They did not designate either species as the genotype. In 1926 Chatton and Lwoff published a preliminary diagnosis, again without illustrations, of a third species of *Hypocomides*, *H. zyrphaeae*, from *Zirfaea crispata* (L.). It was largely on the basis of their description of *H. zyrphaeae* that Raabe (1938) was led to suppose that one of the ciliates which he found parasitizing the gills of *Mytilus edulis* was *H. mytili*. He could not identify on this ciliate, however, a structure to which Chatton and Lwoff referred as the "vestige de frange adorale" and which according to them is in *H. mytili* "constitué seulement par une dizaine de grands cils." This "vestige de frange adorale" is supposed to be better developed in *H. modiolariae* than in *H. mytili*. In *H. zyrphaeae* it is represented, according to Chatton and Lwoff, by "une touffe de cils." The position of the "vestige de frange adorale" in relation to other ciliary structures in these three species of *Hypocomides* is entirely unclear.

On a ciliate which I have studied from *Mytilus edulis* from San Francisco Bay and which conforms in most respects to the description given by Raabe of the form considered by him to be *H. mytili*, I have been unable to detect such a "vestige de frange adorale." The brief notes on the morphology of *H. mytili* to be found in the papers of Chatton and Lwoff in which mention is made of this species (1922a, 1922b, 1924) are not entirely consistent, and it is altogether possible that, as Raabe has pointed out, these authors used the term "vestige de frange adorale" to indicate only a short segment of the distal portion of one of the longer ciliary rows on the right side of the body, which Raabe suggested may be homologous with the two rows bordering the peristomal groove of ciliates of the family Ancistrumidae. It is quite evident from the brief description of *H. zyrphaeae*, however, that the "vestige de frange adorale" of this species is entirely separate from the two long rows on the right. Perhaps *H. mytili* and *H. modiolariae* are not actually congeneric with *H. zyrphaeae*, but this remains to be seen. At any rate, unless it can be established with certainty that the ciliate described by Chatton and Lwoff as *Hypocomides mytili* is not identical with the species thought by Raabe to be *H. mytili*, it seems best to consider the form studied by Raabe to be *H. mytili* and to refer related forms to the same genus.

In the present paper I will give a description of the ciliate from *Mytilus edulis* which I consider to be *H. mytili*, and will add three new species to the genus *Hypocomides: H. botulae* sp. nov. and *H. parva* sp. nov., from the gills and palps of the rock-boring pelecypod, *Botula californiensis* (Philippi), and *H. kelliae* sp. nov., from *Kellia laperousii* Deshayes, a small nestling clam which is frequently encountered in the excavations made in rocks by other molluscs. Another very interesting ancistrocomid ciliate from *Botula californiensis* will be described herein as *Insignicoma venusta* gen. nov., sp. nov.

HYPOCOMIDES MYTILI CHATTON AND LWOFF

(Fig. 1; Plate I, Fig. 1)

The body is elongated and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior three-fifths of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior portion of the left margin is usually not quite so rounded as the right margin, and appears typically to be weakly indented. The body is widest near the middle and rounded posteriorly. Thirty living individuals taken at random ranged in length from 34 μ to 48 μ, in width from 16 μ to 22 μ, and in thickness from 13 μ to 18 μ, averaging about 40 μ by 18 μ by 14.5 μ.

The anterior end of the body is provided with a short contractile tentacle which enables the ciliate to attach itself to the epithelial cells of the gills and palps of the host and to suck out their contents. This tentacle is continuous with an internal tubular canal which can usually be traced in fixed specimens stained with iron hematoxylin for about one-half the length of the body. The canal nearly always appears to be widest in its anterior portion and to be directed obliquely toward the right side of the body as it extends posteriorly.

The cilia of *H. mytili* are about 9 μ in length and are markedly thigmotactic, especially near the anterior end of the body. The ciliary system consists of three separate complexes. The central complex, occupying the middle and right portions of the ventral anterior concavity, consists of seven rows, the one nearest the right being the shortest (one-third to two-fifths the length of the body), the other rows becoming progressively longer toward the left. The sixth and seventh rows are usually approximately one-half the length of the body; in some specimens the seventh row is appreciably shorter than the sixth. To the right of the central complex are two long rows, each about one-half the length of the body. Both these rows originate on the dorsal surface close to the left margin a short distance behind the level of origin of the rows of the central complex and curve ventrally as they extend posteriorly. To the left of the central complex is a series of eight rows which usually are more closely-set than those making up the central complex. The innermost row, which originates on the left margin of the body near the base of the suctorial tentacle, is the shortest, and terminates at a point about one-third the distance from the anterior end of the body to the posterior end. The remaining rows become progressively longer and originate progressively more dorsally and posteriorly, curving ventrally as they extend backward. The outer row of this complex is usually the longest and terminates at a point nearly opposite the point of termination of the outer of the two rows constituting the complex on the right.

According to Raabe, the three ciliary complexes of *H. mytili* are much more distinctly separated than I have observed them to be. Raabe also stated that variations in the number of ciliary rows in the central and left complexes are correlated with two well-differentiated size races: form *minor* (17 μ to 26 μ in length) and form *major* (26 μ to 36 μ in length). I have noted no tendency for the ciliates I consider to belong to this species to be segregated into distinct size races and have seen no examples of *H. mytili* which were in life as small as those assigned to the form *minor* by Raabe. Although I have observed few deviations from the typical number of ciliary rows, it may be of interest to record here the fact that frequently some of the rows of the left complex, particularly the first and second rows, do not stain as well with hematoxylin or impregnate as well with activated silver albumose (protargol) as do the rows of the central and right complexes, and hence may easily escape detection.

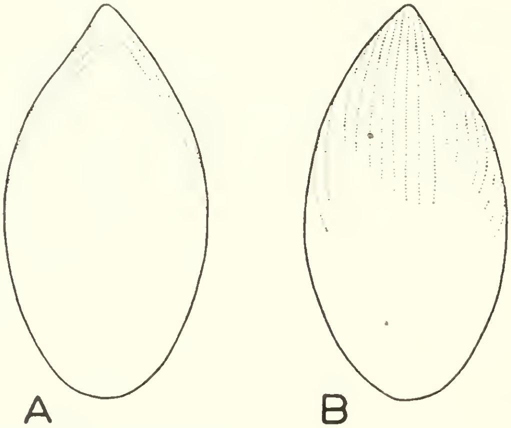

FIGURE 1. *Hypocomides mytili* Chatton and Lwoff. Distribution of ciliary rows, somewhat diagrammatic.[1] A, dorsal aspect; B, ventral aspect.

The cytoplasm is colorless and contains numerous lipoid droplets in addition to food inclusions. Several larger food vacuoles are sometimes observed in the posterior part of the body. The contractile vacuole is centrally located[2] and opens to the exterior on the ventral surface. I have observed no permanent opening in the pellicle.

The macronucleus is usually ovoid, sometimes sausage-shaped; more rarely it is spherical, although Raabe considered the macronucleus of *H. mytili* to be typically spherical. It is situated dorsally in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. In fifteen individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction the macronucleus ranged in length from 9 μ to 13.2 μ and in width from 4.4 μ to 6.9 μ.

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[1] All text figures illustrating this paper are based on camera lucida drawings of individuals fixed in Schaudinn's fluid and impregnated with activated protein silver (protargol).

[2] Raabe stated that the contractile vacuole of *H. mytili* is to be found in a vacuolated area in the posterior part of the body behind the macronucleus. Perhaps his observation was based on specimens which were undergoing degenerative vacuolization and in which the contractile vacuole was not conspicuous. In all normal individuals which I have examined its position was central.

The spherical micronucleus is ordinarily situated near the middle of the body anterior to the macronucleus, although sometimes it is seen to lie to one side of the macronucleus. The chromatin appears in most fixed and stained specimens to be homogeneous, although in some it is aggregated into vague peripheral granules or strands. In fifteen individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.7 μ to 4 μ.

I found *Hypocomides mytili* to be present in large numbers on the gills and palps of about 80 per cent of the specimens of *Mytilus edulis* which I examined from various localities in San Francisco Bay. It is sometimes the only ciliate infesting the mussels, but more commonly it is associated with *Crebricoma carinata* (Raabe) and *Ancistruma mytili* (Quennerstedt).

Hypocomides mytili Chatton and Lwoff

Diagnosis: Length 34 μ–48 μ (according to Raabe 17 μ to 36 μ), average about 40 μ; width 16 μ–22 μ, average about 18 μ; thickness 13 μ–18 μ, average about 14.5 μ. The central ciliary complex is composed of seven rows (according to Raabe seven or eight rows) which are one-third to one-half the length of the body, becoming progressively longer toward the left side; the right complex consists of two rows, each about one-half the length of the body; the left complex consists of eight closely-set rows (according to Raabe five [?] or six rows) one-third to one-half the length of the body. The contractile vacuole is central and opens to the exterior on the ventral surface. The macronucleus is typically ovoid or sausage-shaped, rarely spherical. The micronucleus is spherical. Parasitic on the epithelium of the gills and palps of *Mytilus edulis* L. (Roscoff [Chatton and Lwoff]; Hel [Raabe]; San Francisco Bay, California).

HYPOCOMIDES BOTULAE SP. NOV.

(Fig. 2; Plate 1, Fig. 2)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior one-half of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior portion of the left margin is usually less rounded than the right margin and appears typically to be weakly indented. The body is widest near the middle and rounded posteriorly. Twenty living individuals taken at random ranged in length from 31 μ to 39 μ, in width from 14 μ to 17 μ, and in thickness from 12 μ to 14 μ, averaging about 33 μ by 15 μ by 13 μ.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal which can usually be traced in fixed specimens stained with iron hematoxylin for a distance equal to about three-fifths the length of the body.

In most individuals the canal appears to be directed obliquely toward the right side of the body.

The cilia are about 8 μ to 9 μ in length and are markedly thigmotactic, particularly near the base of the suctorial tentacle. The ciliary system consists of three separate complexes. The central complex, occupying the larger part of the ventral anterior concavity, consists of eleven rows about one-half the length of the body which become progressively longer toward the left side. To the right of the central complex are two longer rows which originate on the dorsal surface close to the right margin a little behind the level of origin of the rows of the central complex and curve downward as they extend posteriorly. Each of the two rows is about three-fifths the length of the body. To the left of the central complex is a series of eleven rather closely-set rows which originate on the left lateral margin and the dorsal surface on the left side and curve ventrally as they extend posteriorly. These rows are about one-half the length of the body and originate and terminate progressively more posteriorly.

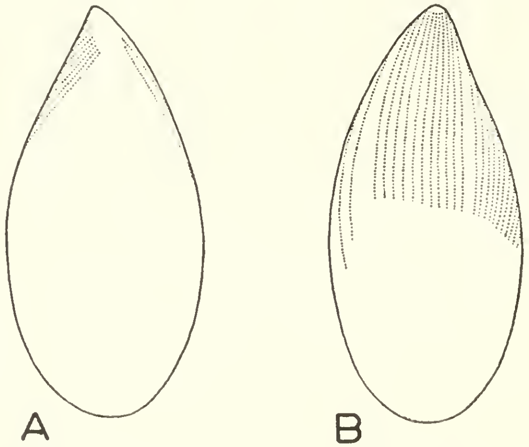

FIGURE 2. *Hypocomides botulae* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

The cytoplasm is colorless and contains numerous lipoid droplets in addition to food inclusions. I have not seen any large food vacuoles in this species. The contractile vacuole, situated near the middle of the body, opens to the exterior on the ventral surface.

The macronucleus is ovoid to sausage-shaped and is situated in the posterior half of the body. Its longitudinal axis is usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin is aggregated into a dense reticulum. Vacuole-like clear spaces of varying size are sometimes apparent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 9 μ to 13 μ and in width from 4.3 μ to 7 μ.

The micronucleus is spherical and is situated dorsally near the middle of the body. The chromatin appears to be homogeneous in fixed specimens. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.4 μ to 3.2 μ.

Hypocomides botulae was present in small numbers on the gills and palps of twelve of the thirty-four specimens of *Botula californiensis* which I examined from localities near Moss Beach, California. It is sometimes found in association with *Hypocomides parva* and *Insignicoma venusta*. No specimens of *Botula falcata* (Gould) which I examined from the same localities were parasitized by these or any other ciliates.

Hypocomides botulae sp. nov.

Diagnosis: Length 31 μ–39 μ, average about 33 μ; width 14 μ–17 μ, average about 15 μ; thickness 12 μ–14 μ, average about 13 μ. The central ciliary complex consists of eleven rows each about one-half the length of the body; the right complex is composed of two rows about three-fifths the length of the body; the left complex consists of eleven closely-set rows about one-half the length of the body. The macronucleus is ovoid to sausage-shaped. The micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

HYPOCOMIDES PARVA SP. NOV.

(Fig. 3; Plate I, Fig. 3)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior two-fifths of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is usually not so rounded as the right margin, and appears typically to be nearly straight or weakly indented. The body is widest near the middle and rounded posteriorly. Twenty-five living individuals taken at random ranged in length from 21 μ to 29 μ, in width from 10 μ to 13 μ, and in thickness from 8 μ to 11 μ, averaging about 26 μ by 12 μ by 10 μ.

The anterior end of the body is provided with a short contractile suctorial tentacle continuous with an internal tubular canal. The canal is usually directed obliquely toward the right side and can be traced in most fixed specimens stained with iron hematoxylin for about one-half the length of the body.

The cilia of *Hypocomides parva* are about 6 μ to 7 μ long and are strongly thigmotactic, particularly near the base of the suctorial tentacle. The ciliary system consists of three separate complexes. The central complex, which occupies the larger part of the concave depression on the anterior part of the ventral surface, consists of eight rows which are about two-fifths the length of the body. To the right of this complex are two longer rows which originate on the dorsal surface close to the right margin behind the level of origin of the rows that constitute the central complex. Each of these rows is about three-fifths the length of the body and curves ventrally as it extends posteriorly. The outer row originates and terminates the more posteriorly. To the left of the central complex is a series of eight rows which originate on the left lateral margin and on the left side of the dorsal surface and curve ventrally as they extend backward. These rows are about two-fifths the length of the body and originate and terminate progressively more posteriorly. The outermost row, however, is somewhat shorter than the other rows and is usually seen to terminate a little anterior to the point of termination of the seventh row.

The cytoplasm is colorless and contains numerous small lipoid droplets and food inclusions. I have distinguished no large food vacuoles in this species. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is typically ovoid, sometimes spherical, rarely sausage-shaped. It is situated in the posterior half of the body with its longitudinal axis usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing a few vacuole-like clear spaces of varying size. These are most prominent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 4.2 μ to 8.2 μ and in width from 4.2 μ to 5.3 μ.

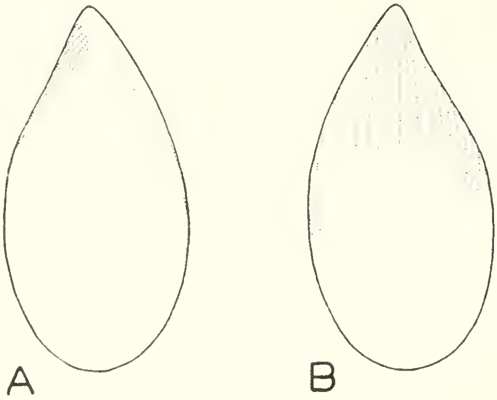

FIGURE 3. *Hypocomides parva* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

The micronucleus is usually spherical and is situated dorsally a short distance anterior to or to one side of the macronucleus. Sometimes it is located as far anteriorly as the middle of the body. In fixed and stained preparations the chromatin of the micronucleus appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 1.9 μ to 2.3 μ.

Hypocomides parva was present on the gills and palps of nineteen of the thirty-four individuals of *Botula californiensis* which I examined from localities near Moss Beach, California. It is sometimes associated with *H. botulae* and *Insignicoma venusta*. In my experience it is the most common species of ciliate parasitizing this mollusc.

Hypocomides parva sp. nov.

Diagnosis: Length 21 μ–29 μ, average about 26 μ; width 10 μ–13 μ, average about 12 μ; thickness 8 μ–11 μ, average about 10 μ. The central ciliary complex comprises eight approximately equal rows about two-fifths the length of the body;

the right complex consists of two rows about three-fifths the length of the body; the left complex consists of eight rows, each about two-fifths the length of the body, the eighth row being usually somewhat shorter than the others. The macronucleus is typically ovoid. The micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

HYPOCOMIDES KELLIAE SP. NOV.

(Fig. 4; Plate I, Fig. 4)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior one-third of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is not as rounded as the right margin, and typically is nearly straight or weakly indented. The body is widest near the middle and rounded posteriorly. Twenty living individuals taken at random ranged in length from 31 μ to 37 μ, in width from 13 μ to 15 μ, and in thickness from 11 μ to 13 μ, averaging about 33 μ by 14 μ by 12 μ.

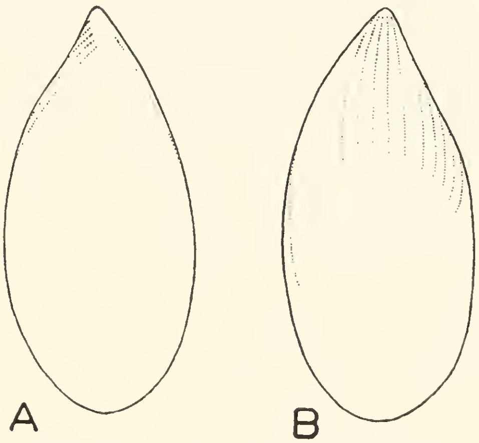

FIGURE 4. *Hypocomides kelliae* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The canal is usually directed toward the right side of the body as it extends posteriorly. It can be traced in most fixed individuals stained with iron hematoxylin for about three-fifths the length of the body.

The thigmotactic cilia of *H. kelliae* are about 8 μ or 9 μ in length. The ciliary system is composed of three separate complexes. The central complex, occupying the larger part of the anterior ventral depression, consists of five equal rows about one-third the length of the body. To the right of this system is a single long row about two-thirds the length of the body. This row originates on the dorsal surface close to the right margin a little behind the level of origin of the central rows and curves ventrally as it extends posteriorly. To the left of the central complex is a series of five rows, one-third the length of the body, which originate on the left lateral margin or dorsal surface on the left side and curve ventrally as they extend posteriorly. These rows originate and terminate progressively more posteriorly.

The cytoplasm is colorless and contains numerous small lipoid droplets and food inclusions. I have observed no large food vacuoles in this species. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid or sausage-shaped, usually about two times as long as wide. It is situated in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be organized into a very dense reticulum which sometimes is seen to enclose vacuole-like clear spaces of varying size. These are more evident near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 7.8 μ to 14 μ and in width from 3.9 μ to 7 μ.

The micronucleus varies in shape from spherical to ovoid; typically it is ovoid. It is usually situated dorsally a short distance anterior to the middle of the body or to one side of the anterior part of the macronucleus. In most fixed and stained individuals of *H. kelliae* the chromatin of the micronucleus is aggregated into deeply-staining peripheral strands. In ten specimens fixed in Schaudinn's fluid and stained by the Feulgen reaction the micronucleus ranged in size from 1.9 μ by 1.5 μ to 2.3 μ by 1.9 μ.

Hypocomides kelliae was present in nine of the twenty-eight individuals of *Kellia laperousii* which I examined from localities near Moss Beach, California. Also associated with this mollusc is a small ancistrumid ciliate which it may be possible for me to describe in a subsequent paper.

Hypocomides kelliae sp. nov.

Diagnosis: Length 31 μ–37 μ, average about 33 μ; width 13 μ–15 μ, average about 14 μ; thickness 11 μ–13 μ, average about 12 μ. The central ciliary complex consists of five rows about one-third the length of the body; the right complex consists of a single row about two-thirds the length of the body; the left complex consists of five rows about one-third the length of the body. The macronucleus is ovoid or sausage-shaped. The micronucleus is typically ovoid. Parasitic on the epithelium of the gills and palps of *Kellia laperousii* Deshayes (Moss Beach, California). Syntypes are in the collection of the author.

INSIGNICOMA VENUSTA GEN. NOV., SP. NOV.

(Fig. 5; Plate I, Fig. 5)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The anterior one-half of the ventral surface, on which the major part of the ciliary system is disposed, is weakly concave; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is usually not so rounded as the right margin and typically is nearly straight or slightly indented. The body is widest near the middle and rounded posteriorly. Twenty-five living individuals taken at random ranged in length from 42 μ to 52 μ, in width from 18 μ to 21 μ, and in thickness from 15 μ to 18 μ, averaging about 48 μ by 20 μ by 17 μ.

The anterior end of the body is provided with a contractile suctorial tentacle continuous with an internal tubular canal. In most fixed specimens stained with iron hematoxylin the canal can be traced for about three-fifths the length of the body. It usually appears to be directed obliquely toward the right side.

The ciliary system consists of four separate complexes. Two long, widely-spaced rows on the right side of the body originate on the dorsal surface close to the right margin at the anterior end and are about two-thirds the length of the body. They curve ventrally as they extend posteriorly. A central complex of fourteen or fifteen rows occupies the larger part of the ventral anterior depression. These rows are on the average about one-half the length of the body and originate progressively more posteriorly toward the left side. The outer two or three rows on the left, however, do not usually terminate quite so far posteriorly as the twelfth row. The rows of this complex are usually a little more closely-set on the right side than on the left. To the left of the central complex is a series of sixteen or seventeen rows about one-half the length of the body which with the exception of the outer three or four rows are very closely-set. The innermost row originates on the left lateral margin near the base of the suctorial tentacle; the remaining rows originate progressively more dorsally and posteriorly. The distal portions of several of the inner rows of this complex are usually visible on the left side of the ventral surface. Posterior to the middle of the body on the left side is a nearly V-shaped series of cilia which originates

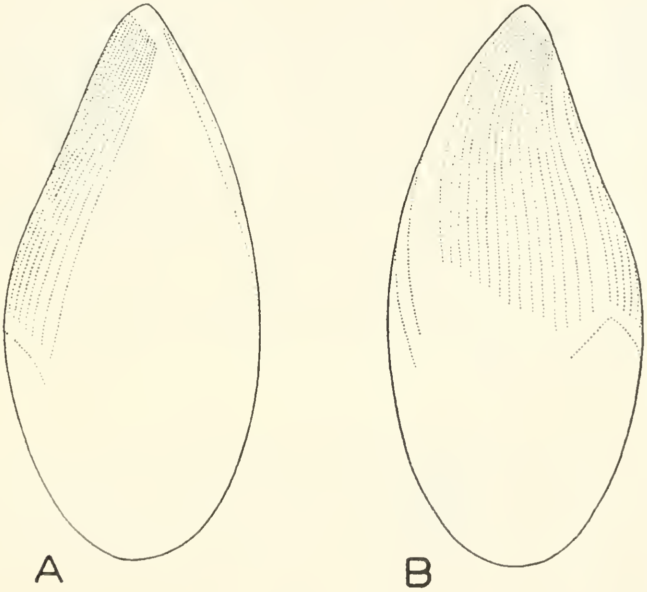

FIGURE 5. *Insignicoma venusta* gen. nov., sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

on the ventral surface in the posterior third of the body, extends anteriorly and to the left to a point a short distance behind the distal portion of the gap separating the central and left ciliary complexes, then bends abruptly backward and dorsally. The cilia of this fourth complex are about 12 μ to 14 μ in length. The cilia of the other rows are about 8 μ or 9 μ in length and strongly thigmotactic, especially near the base of the suctorial tentacle.

The cytoplasm is colorless and contains numerous small lipoid droplets in addition to food inclusions. A few larger food vacuoles are sometimes observed near the posterior end. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid or sausage-shaped and is situated in the posterior half of the body with its longitudinal axis usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying sizes. These are most prominent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 12 μ to 17 μ and in width from 4.4 μ to 9 μ.

The spherical micronucleus is commonly situated dorsally a short distance anterior to or to one side of the macronucleus. In fixed and stained preparations the chromatin appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.4 μ to 4 μ.

Insignicoma venusta was found to parasitize the gills and palps of nine of the thirty-four specimens of *Botula californiensis* which I collected at localities near Moss Beach, California.

Insignicoma gen. nov.

Diagnosis: The body is elongated and somewhat flattened dorso-ventrally. The anterior end of the body is narrowed and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary system consists of four separate complexes. The central complex, occupying the major portion of the shallow concavity on the anterior one-half of the ventral surface, is bounded on the right by a small number of widely-spaced rows which curve ventrally as they extend posteriorly; to the left of the central complex is a series of closely-set rows about one-half the length of the body which originate progressively more posteriorly on the left lateral margin and dorsal surface on the left side and curve ventrally as they extend backward; the fourth complex consists of a V-shaped series of long cilia which lies immediately behind the distal portion of the gap separating the central and left ciliary complexes. The contractile vacuole is central and opens to the exterior

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PLATE I

All figures have been drawn with the aid of a camera lucida from specimens fixed in Schaudinn's fluid and stained with iron hematoxylin. × 1870.

FIGURE 1. *Hypocomides mytili* Chatton and Lwoff. Ventral aspect.

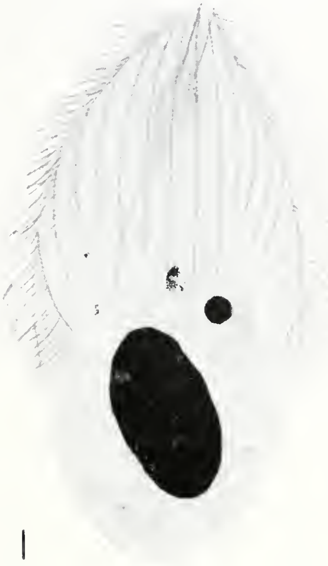
FIGURE 2. *Hypocomides botulae* sp. nov. Ventral aspect.

FIGURE 3. *Hypocomides parva* sp. nov. Ventral aspect.

FIGURE 4. *Hypocomides kelliae* sp. nov. Ventral aspect.

FIGURE 5. *Insignicoma venusta* gen. nov., sp. nov. Ventral aspect.

PLATE I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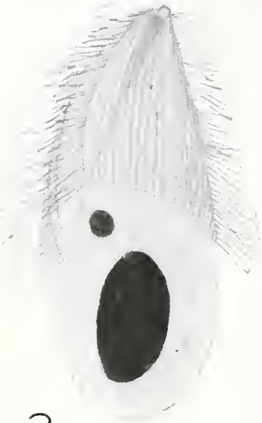
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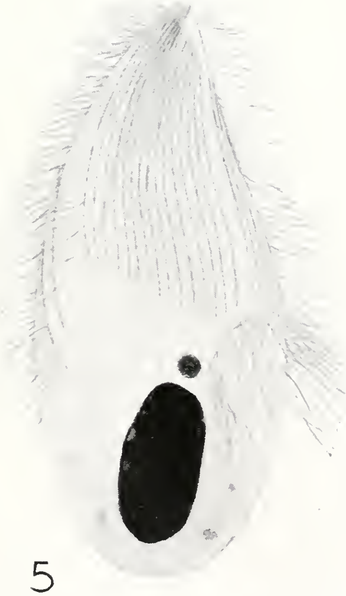
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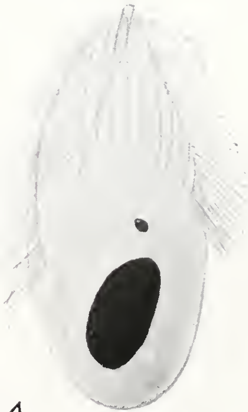
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on the ventral surface; there is no permanent opening in the pellicle. Genotype: *Insignicoma venusta* gen. nov., sp. nov.

Insignicoma venusta gen. nov., sp. nov.

Diagnosis: Length 42 μ–52 μ, average about 48 μ; width 18 μ–21 μ, average about 20 μ; thickness 15 μ–18 μ, average about 17 μ. The central ciliary complex consists of fifteen (rarely fourteen) rows about one-half the length of the body which originate progressively more posteriorly toward the left side; the right complex consists of two widely-spaced rows about two-thirds the length of the body which originate on the dorsal surface close to the left margin and curve ventrally as they extend posteriorly; the left complex consists of sixteen or seventeen closely-set rows about one-half the length of the body which originate progressively more posteriorly on the left margin and dorsal surface on the left side and curve ventrally as they extend posteriorly; the V-shaped series of cilia constituting the fourth complex originates on the ventral surface in the posterior third of the body, extends anteriorly and to the left to a point a short distance behind the distal portion of the gap separating the central and left ciliary complexes, then bends abruptly backward and dorsally. The cilia of the fourth complex are approximately 12 μ–14 μ in length; those of the other three complexes are approximately 8 μ–9 μ in length. The macronucleus is ovoid or sausage-shaped; the micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

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NATURAL HETEROAGGLUTININS IN THE BODY–FLUIDS AND SEMINAL FLUIDS OF VARIOUS INVERTEBRATES[1]

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INTRODUCTION

Since the early work of Landois (1875) numerous investigators have noted in the normal serum of various species of animals, particularly among the vertebrates, the occurrence of agglutinins that act on the cells of certain other species (cf. Wiener, 1943; Landsteiner, 1945; Thomsen, 1932). Such natural heteroagglutinins have also been frequently reported to occur in the serum or body-fluids of various invertebrates (see literature in Huff, 1940; Tyler and Metz, 1945). Heteroagglutination reactions are frequently encountered in fertilizin studies and have been reported by several investigators (Lillie, 1913, 1919; Glaser, 1914; Just, 1919, 1930; Sampson, 1922; Godlewski, 1934; Hartmann *et al.,* 1940; Runnström *et al.,* 1944) as occurring between spermatozoa and foreign egg-water preparations, body-fluids and spermatozoa or their extracts. Further information concerning the range and nature of the heteroagglutination reactions is of importance, then, in analysis of problems of fertilization particularly in regard to the specificity and role of the interacting substances that are obtained from eggs and sperm.

A study of heteroagglutination reactions with lobster serum (Tyler and Metz, 1945; Tyler and Scheer, 1945), which normally acts on the sperm or the blood-cells of a wide variety of species throughout the animal kingdom, showed that at least ten distinct relatively class-specific agglutinins are present in the serum of this species. This was determined by means of absorption tests. In the controls for those tests the supernatant fluids of the sperm suspensions used for absorption were also examined for agglutinating activity and it was found, particularly when the sperm had not been previously washed, that the fluids from sperm of some species were active on cells of certain other species. Tests were then made with the body-fluids and these, too, were found to be active.

The present paper reports the results of this examination of the body-fluids and the sperm-supernatants of various species of invertebrates for the occurrence of heteroagglutinins. The species examined were for the most part those that have been used or are potentially useful in fertilizin studies. In addition a few absorption tests were made with starfish body-fluid to determine whether or not its agglutinating activity is attributable to the presence of several heteroagglutinins, each with broad specificity such as was found in lobster serum.

MATERIALS AND METHODS

The body-fluids of twelve species of animals among the annelids, echinoderms, mollusks and tunicates were examined for possible agglutinating action on sperm suspensions of various species of invertebrates and, in a few cases, on erythrocyte suspensions of various vertebrates. The body-fluids were obtained by incision into the body cavity or by insertion of a hypodermic syringe into the body cavity. In the case of Ciona the fluid was obtained directly from the heart. The fluids were clarified by centrifugation and tested in the manner previously described (Tyler and Metz, 1945). The supernatant fluids of sperm suspensions of eleven of the same species were also tested. These were obtained by centrifugation of approximately 10 to 20 per cent suspensions of "dry" sperm in sea water. They may be termed diluted seminal fluids.

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[1] This work has been aided by a grant from the Rockefeller Foundation. I am indebted to Miss Margaret L. Campbell for technical assistance.

EXPERIMENTAL PART

In all of the species that were examined the body-fluids were found to possess agglutinating activity for the cells of certain other species. The results are presented in Table I. In some species (e.g., 1, 6, 18, 19, 21) the fluids exhibited agglutinating action on the cells of most of the species that were tested. However, the fluids of

TABLE I

Heteroagglutinating action of body-fluid (b) and seminal fluid (s) of various invertebrates

Spermatozoa (species 1 to 25) or erythrocytes (species 26–34) of:	Fluids of:																						
	1		4		6	12		13		14		16		18		19		21		22		23	
	b	s	b	s	b	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s
POLYCHAETS																							
1. *Chaetopterus variopedatus*	0	0	0	0	+	0	0	+	+	+	+	+	+	+	+	+	+	+		0	0	0	0
2. *Halosydna johnsoni*		0	0	0	+	0	0	+	+	+	+			+	+								
3. *Sabellaria californica*	+	+	0	0		0	0	0	0	0	0	0		+		+	+	0				0	0
ECHIUROIDS																							
4. *Urechis caupo*	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+		+		+	
5. *Thalassema* sp.	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+		+			
AMPHINEURANS																							
6. *Mopalia muscosa*	+	+	0	0	0	+	0	+	+	0	0	0	0	+	+		+	0		+		+	+
7. *Ischnochiton magdalensis*	+	+	0	0	0	+		+	+	0	0	0		+	+		+	0		+			+
GASTROPODS																							
8. *Acmea digitalis*	+	+	+		0	0				0	0			+	0			0				+	+
9. *Lottia gigantea*	+	+	+	0	0	0	0	+		0				+				0		+			
10. *Tegula galena*	+	+	+		+	0					0			+	+			+		+			
11. *Astraea undosa*	+	+			+	0		+	0	0				+	+			+		+			+
12. *Megathura crenulata*	+	+	+	0	+	0	0	+	+	0	0	0	0	+	+	+		+		+		+	+
PELECYPOD																							
13. *Mytilus californianus*	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+		+		+		+	
ECHINOIDS																							
14. *Strongylocentrotus purpuratus*	0	0	0	0	+	0	0	+	+	0	0	0	0	0	0	0	0	+	0		0	0	0
15. *S. franciscanus*					+	0		+			0	0	0	0	0								
16. *Lytechinus pictus*	0	0	0	0	+	0	0	+	+	0	0	0	0	0	0	0		+		0		0	
17. *Dendraster excentricus*		0	0	0	+	0			+	0	0		0	0	0					0			

TABLE I—*Continued*

Spermatozoa (species 1 to 25) or erythrocytes (species 26–34) of:	Fluids of:																						
	1		4		6	12		13		14		16		18		19		21		22		23	
	b	s	b	s	b	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s
ASTEROIDS																							
18. *Patiria miniata*	+	+	0	0	0	+	+	0	0	0	0	0	0	0	0	0			+	0	0	0	0
19. *Pisaster ochraceus*	+	+	0	0	0	+	+	0	0	0	0	0	0	0	0	0	0	+		0	0		0
20. *Astropecten armatus*		+		0					0	0	0	0	0	0	0								
HOLOTHURIOID																							
21. *Stichopus californicus*			0			0		0		0		0		0	0	0		0	0				
ASCIDIANS																							
22. *Ciona intestinalis*	+	+	0	0	+	0	0	0	0	0	0	0	0	+	+	+	+	+		0	0	0	0
23. *Styela barnharti*	+	+	0	0	+	0		0	0	0	0	0	0	+	+	+	+	+	+	0	0	0	0
24. *Ascidia ceratodes*		+	0	0		0		0		0				+	+			+		0	0	0	0
FISH																							
25. *Leuresthes tenuis*	0		0			0		0		0	0		0	0	0			+	+		+		
26. *Girella nigricans*	0								0	0	0		0	0	0		0	+	+				
AMPHIBIA																							
27. *Rana catesbiana*	+	+																					
28. *Bufo halophilus*		+		0		0			0		0				0		0		0		+		+
REPTILE																							
29. *Sceloporus occidentalis*	+	0									0	0			0	0		+	+		0		
BIRD																							
30. Chicken		+		0			0		+		0			+	0		0		+		0		0
MAMMALS																							
31. Guinea pig								+	+	0				+									
32. Rabbit									+		0												
33. Sheep		+		0			0	+	+		0		0	+							0		0
34. Man									+		0			+									

none of the twelve species examined were found to possess agglutinating action on all of the species that were tested.

With one exception no heteroagglutination reactions occurred with body-fluids and cells of animals belonging to the same taxonomic class. The exception to this consists in the agglutination of Sabellaria sperm by Chaetopterus fluid. It is of interest in this connection that these two genera are placed (Pearse, 1936) in separate subclasses (cryptocephala and phanerocephala respectively) of the polychaets. Sabellaria also differs from the other two polychaets that were tested in that its cells fail to agglutinate in the fluid of the mussel (13), sea-urchins (14, 16) and sea cucumber (21) as well as that of the lobster previously (Tyler and Metz, 1945) reported.

Another feature of the results is that closely related species behave alike with respect to the ability or inability of their cells to be agglutinated by the various body-fluids. This was evident in the previously reported experiments with lobster serum.

In the present data differences are observed between species belonging to different orders, as in the case of the two species (8 and 9) belonging to the docoglossid gastropods which differ from the three rhipidoglossid species (10, 11, and 12) in their reactions to two of the fluids (6 and 21). With the other twenty-nine species tested, similarity in behavior is exhibited by members of the same class or sub-class.

The tests with the diluted seminal fluids gave results that in most cases paralleled those obtained with the body-fluids. Eight exceptions (1 on 29; 4 on 9 and 12; 12 on 6; 13 on 11; 18 on 8 and 30; 21 on 14) may be noted in Table I out of a total of 186 cross-combinations in which both seminal fluid and body-fluid were examined. These exceptions are all in the same direction; namely, a failure of the dilute seminal fluid to cause agglutination while the corresponding body-fluid is active. It can, then, be stated that in those cases in which the diluted seminal fluid is found to possess heteroagglutinating activity, the corresponding body fluid is likewise found to be active. Thus, similarly acting heteroagglutinins are found in both body-fluid and seminal fluid. The above-mentioned few exceptions may be attributed to failure to obtain, in certain seminal fluid preparations, sufficient concentration of a particular heteroagglutinin to produce a visible reaction with the cells of some species. However, the tests necessary to determine the validity of this explanation have not, as yet, been made.

From the similarity in action of seminal fluid and body-fluid, it might be inferred that the activity of the former is due to contamination with the latter. However, it may be noted that in most of the species employed for preparation of seminal fluid (e.g., 4, 12, 14, 16, 18, 19, 21, 22) the sperm is readily obtained without any appreciable admixture of body-fluid. Another interpretation is that seminal fluid is normally similar to body-fluid in composition. Against this may be cited the fact that readily recognizable constituents of body-fluid, such as hemocyanin in the mollusks, are not observed in the seminal fluids. A third possibility is that identical heteroagglutinins are present in both fluids. However, serological similarity does not imply entirely identical molecular constitution. Reaction with a specific antigen implies similarity only on the part of the specific combining groups of the antibodies from diverse sources. In the present case it has not been shown that the heteroagglutinin in seminal fluid and that in body-fluid both react with the same antigenic group or structure on the sperm that they agglutinate. However, the generally parallel behavior of the two fluids, when tested with spermatozoa of different species, favors that view.

Absorption tests, to determine whether or not more than one heteroagglutinin is involved in the action of a particular fluid, were carried out with Patiria body-fluid. These were done in the manner previously described (Tyler and Metz, 1945). Before being used for absorption the sperm were washed repeatedly in order to free them of agglutinins contributed by the seminal fluid, and this was checked in each test by examination of the supernatant of an aliquot part of the sperm for agglutinating activity.

Samples of Patiria body-fluid were absorbed with sperm of six species of animals and tested for agglutinating activity on sperm of nine species. The results are given in Table II. This limited set of tests reveals the presence of at least four distinct heteroagglutinins in Patiria body-fluid. These evidently comprise:—one for the two polychaets, one for the two echiuroids and Mytilus, one for the two gastropods, and one for the two ascidians. It seems likely, then, that the situation in Patiria body-fluid is similar to that previously reported for the lobster; namely, the presence of a number of distinct agglutinins, each with broad group specificity.

Discussion

Heteroagglutinins are evidently normal constituents of the body-fluids of animals. They have generally been considered to be non-specific agents. However, from the fact that the fluids of various species act on the cells of different assemblages of other species, the heteroagglutinins must be regarded as having some degree of specificity. The previously reported (Tyler and Metz, 1945) absorption tests with lobster-serum gave evidence of the presence of ten distinct heteroagglutinins which are, for the most part, each specific for a taxonomic class of animals. The present results with Patiria body-fluids are indicative of similarly broad specificity on the part of the four heteroagglutinins found therein. It is clear, however, that heteroagglutinins of different species and also different heteroagglutinins of the same animal may differ considerably in the range of species on which they act. The rule is that closely related species react alike to a particular fluid, but the closeness of relationship required depends upon the particular heteroagglutinating fluid employed. In the present tests (Table I) we find that species that belong to the same class, in most instances, behave alike with respect to the ability or inability of their cells to be agglutinated by the body-fluids of all twelve of the species examined.

TABLE II

Agglutinative activity of Patiria body-fluid after absorption with spermatozoa of various species

Spermatozoa of:	Body-fluid absorbed with washed sperm of:					
	Chaetopterus	Urechis	Thalassema	Astraea	Megathura	Mytilus
Chaetopterus	0	+	+	+	+	+
Halosydna	0	+	+	+	+	+
Urechis	+	0	0	+	+	0
Thalassema	+	0	0	+	+	0
Astraea	+	+	+	0	0	+
Megathura	+	+	+	0	0	+
Mytilus	+	0	0	+	+	0
Ciona	+	0	0	+	+	+
Ascidia	+	0	0	+	+	+

In the sera of various mammals natural heteroagglutinins are found (cf. Thomsen, 1932; Wiener, 1943; Landsteiner, 1945) that are relatively species-specific and in human sera, as is well known, natural isoagglutinins are encountered that differentiate groups of individuals. In the various invertebrate body-fluids examined, agglutinins of such specificity have not, as yet, been found, the fluid of a particular species being inactive on cells of closely related species. However, from the ripe gametes, of many of these species natural agglutinins are obtained that act within the species. These consist in the fertilizins from eggs and antifertilizins from sperm described by Lillie (1913 et seq.), Just (1930), Frank (1939), Tyler (1939 et seq.), Hartman *et al.* (1939 et seq.), Runnström *et al.* (1942 et seq.) and others. While these agents act on gametes of the opposite sex within the species, they also have been found to act on closely related species and in some instances the preparations act on remotely related species. Thus Arbacia egg water was found (Lillie, 1913) to agglutinate Nereis sperm, and sea-urchin eggs have been found (Runnström *et al.*, 1942) to be agglutinated by sperm extracts of animals as remotely related as the salmon and the ox. In Lillie's experiment it was shown that absorption with Nereis sperm removed the cross-reacting substance from Arbacia egg water without diminishing its agglutinating action on Arbacia sperm. Similar absorption experiments have not been reported in most of the cross-heteroagglutination reactions obtained by later workers with fertilizin and antifertilizin preparations. The need for such experiments is quite evident before any adequate determinations can be made of the specificity of these interacting substances obtained from the gametes. The present results may provide a helpful basis of procedure in such experiments.

The bearing of the heteroagglutination reactions on phylogenetic questions has been previously (Tyler and Metz, 1945) discussed. The present results are consistent with the previously expressed view that the reactivity of the cells of a particular species with various fluids is a characteristic of a group of related species and constitutes a group-specific trait in addition to the various group-specific morphological and chemical features of animals. There is no reason, as yet, for considering this trait to be of more general significance than any other in any applications that might be made to phylogenetic problems.

SUMMARY

1. The body-fluids of 12 species of invertebrates (including two ascidians) and the seminal fluids of 11 species were examined for agglutinating action on the spermatozoa or blood cells of 34 species of animals.

2. All of the fluids were found to contain agglutinins for the cells of some of the species tested. Five of the fluids gave reactions with most of the species but none reacted with all of the species.

3. With one exception no heteroagglutination reactions were obtained with fluids and cells of animals belonging to the same taxonomic class.

4. Closely related (same class in most cases or same order in some) species were found to behave alike with respect to the ability or inability of their cells to react to the various fluids, and the fluids of closely related species exhibited similar reactivity.

5. The diluted seminal fluids gave reactions that in most cases paralleled those obtained with the body-fluids.

6. Absorption tests revealed the presence of at least four distinct heteroagglutinins in Patiria body-fluid, and indicated that each is characterized by a broad group-specificity similar to that previously reported for lobster-serum.

7. The general bearing of these results on fertilizin-antifertilizin reactions and on phylogenetic problems is briefly discussed.

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DISTRIBUTION AND PROPERTIES OF INTRACELLULAR ALKALINE PHOSPHATASES[1]

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The chemical and structural composition and the interrelationships of the components of the cell have long been the concern of biologists, in hope of revealing the fundamental processes within the structural and functional unit of all living matter. The early studies of Miescher upon the chemical composition of the nucleus were contemporary with the studies of the structure of the nucleus by van Beneden, Flemming, and others, which later culminated in the firm establishment of the nucleus as the site of phenomena of central importance in the mechanism of Mendelian heredity. The biochemical line of investigation, started by Miescher, has had no such tradition of continuous progress as have the studies of chromosomal structure; but as a result of sporadic advances following the appearance of new methods and techniques, certain facts about the chemical composition of the cell and the nucleus have become firmly established. Miescher noted that the nucleus had a high content of organically bound phosphate; later researches (Levene and Bass, 1931) have fully confirmed this, and have led to a fairly comprehensive knowledge of the chemical composition of nucleic acid, the substance in which all this nuclear phosphate is contained. The unit of nucleic acid is the mononucleotide, a phosphoric acid ester of pentose sugar in glucosidic linkage with one of the purine or pyrimidine heterocyclic bases. The nucleic acids, which have been intensively studied, have been found to be polynucleotides with four different bases, and in their native form they are very highly polymerized, consisting in some cases of hundreds or thousands of nucleotides. There are two types of nucleic acid with respect to the sugar; one contains a pentose which whenever identified has been found to be d-ribose, and the other a desoxypentose found to be d-ribodesose. The wide application of the Feulgen cytochemical test for desoxypentose indicates that nucleic acid with this sugar does not occur outside of the chromatin of the cell nucleus (that is, in the chromosomes), and actual analysis of isolated nuclei and chromosomes has confirmed the view that desoxypentose is the characteristic nucleic acid of chromatin (Mirsky and Pollister, 1942). The name chromonucleic acid has recently been proposed (Pollister and Mirsky, 1943) for desoxypentose nucleic acid as a convenient way of emphasizing this striking limitation in distribution. Nucleic acids can also be located within the cell by means of the intense specific absorption of ultra-violet light by purine and pyrimidine bases, and Caspersson (1936, 1940) has made use of a combination of microspectroscopy and the Feulgen nucleal reaction to determine the distribution of the Feulgen negative pentose nucleic acids. This was found to be largely in the cell cytoplasm, the occurrence of pentose nucleic acid in the nucleus being confined to the plasmosome, or the non-chromatin nucleolus. The term plasmonucleic acid has been proposed to embody this information concerning the distribution of pentose nucleic acid. The nucleic acid makes up but a part, usually less than half, of the content of the nucleus. The remainder is apparently largely protein (Mirsky and Pollister, 1942). Much of this is of the basic type, either histone or protamine, but there is also evidence of protein of globulin type (Caspersson, 1936; Mazia and Jaeger, 1939).

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In view of the fact that the nuclear chromatin contains a large amount of phosphate in desoxyribose nucleic acid, it is of considerable interest that definite evidence of phosphatase activity within chromatin has been found. Almost simultaneously the author (Krugelis, 1942) and Willmer (1942) reported the demonstration of alkaline phosphatase activity within chromosomes. This discovery stimulated the present study, the purpose of which is two-fold. The first purpose was to investigate the occurrence of alkaline phosphatase in all parts of the cell, and to investigate further the correlation of desoxyribose nucleic acid with alkaline phosphatase activity in the chromosomes. The material which seemed most promising for this first part of the study were the salivary gland chromosomes of Drosophila larvae, because of their peculiar structure which manifests itself in bands or regions rich in desoxyribose nucleic acid, alternating with interband regions poor or lacking in desoxyribose nucleic acid. This part of the investigation can be expressed in the form of a question: Is phosphatase activity located in the chromosomal regions rich in desoxyribose nucleic acid? The second part of the investigation followed from the first, and included the use as substrate of actual chemical substances that are found in the cell, in order to discover whether alkaline phosphatase is capable of hydrolyzing naturally-occurring substances with which its activity seems to be physically associated. If the activity of phosphatase is located in regions rich in desoxyribose nucleic acid, is it capable of hydrolyzing desoxyribose nucleic acid and other naturally occurring substances in the cell? This part of the investigation it was hoped would lead to some suggestion as to the possible role of phosphatase activity within the cell and the chromosome.

Methods and Materials

The histochemical method used was that developed by Gomori (1939, 1941); when using smeared insect material it was found necessary to make some modifications of the preliminary steps. This process involved dissecting, and smearing the insect organs in isotonic saline, or Ringer's, made according to Buck and Melland (1942). After smearing, the material was fixed in 95 per cent ethyl alcohol vapors for one hour, then fixed in liquid 95 per cent ethyl alcohol for two hours. Material fixed as short a time as one hour and as long a time as 48 hours showed little appreciable difference in the results. The coverslips fell off readily in the alcohol, and the smears on the slides were washed briefly in distilled water and then incubated in the substrate solution. The mammalian organs used, namely, the kidney, liver, testis, intestine, and pancreas, were removed after ether anesthesia, fixed in 95 per cent ethyl alcohol for 24 hours, dehydrated in 100 per cent ethyl alcohol, cleared in benzene, and embedded in paraffin in the usual manner. Sections of 5 microns thickness were cut. To avoid any variation in mounting or incubating, sections of the five organs were mounted together on one slide. After deparaffinization, hydration, and washing, sections were ready for immersion and incubation in the substrate solution.

The process from this point on was the same for both the sectioned and the smeared materials. The substrate solution was of the following composition:

2 parts of 0.1 M sodium glycerophosphate (or 0.1 M other organic phosphate ester)
2 parts of 0.1 M calcium nitrate
1 part of 0.1 M veronal buffer at pH 9.4
5 parts of distilled water.

The pH was checked with thymol blue and adjusted to pH 9.0 to 9.2 with NaOH. The incubation was carried out at 25–28° C. and lasted from 12 to 24 hours. Following the incubation in the substrate, the slides containing the smears or the sections were immersed in 0.02 M calcium nitrate for one minute, then into 0.1 M cobaltous nitrate for two minutes, then dilute potassium sulfide for two minutes. After this visualization treatment, slides were washed, dehydrated, cleared, and mounted by the usual histological technique. The results were checked, in some cases, by parallel experiments in which the silver nitrate visualization method (Gomori, 1939) was used. When no activity was obtained in the substrate-incubated slides, the reaction was allowed to proceed for 72 hours to verify the negative results.

At the suggestion of Dr. A. E. Mirsky partially depolymerized nucleic acid was tried as the substrate. This depolymerization was accomplished by using desoxyribonuclease from beef pancreas, made according to the method of McCarty (1946). Desoxyribose nucleic acid is dissolved in distilled water and then placed in a nuclease medium of the following composition:

0.01 per cent magnesium sulfate
0.003 M gelatin
0.025 M veronal-HCl buffer at pH 7.5.

The nuclease is allowed to react upon the desoxyribose nucleic acid for 24 hours in which time the solution becomes clear and loses its viscosity. This change is used as a criterion for the depolymerization of the nucleic acid. This depolymerization leaves the nucleic acid in only a less highly polymerized state; and does not, for example, reduce it to an approximation of tetranucleotide. After the depolymerizing reaction, the solution is heated to 60° C. for fifteen minutes to destroy the activity of the nuclease on the desoxyribose nucleic acid, and then is used as the substrate source of phosphate ester.

The biological materials used in this study were insect and mammalian organs. The salivary glands of the larvae of Drosophila were used for the first part of the experimentation. The larvae were raised at 17–18° C. and at room temperature. The glands were dissected out when the larvae were full grown and had left the food prior to pupation. The species of Drosophila investigated included the Brazilian species *Drosophila pallidipennis* obtained through the generosity of Professor T. Dobzhansky, *D. simulans, D. virilis,* and *D. ananassae* obtained from the stocks at the Biological Laboratories, Cold Spring Harbor, N. Y. The major part of the investigation on Drosophila was done on *Drosophila melanogaster*.

The mammalian organs used were those of mice obtained from the stocks maintained at Columbia University by Professor L. C. Dunn and Dr. S. Gluecksohn-Schoenheimer. The testes were used either after being smeared in the body fluid surrounding the tubules, or after being fixed and embedded as the other organs were.

Yeast nucleic acid and adenylic acid were obtained from the Schwartz Laboratories, New York; thymonucleic (desoxyribose nucleic) acid and nuclease were generously supplied by Dr. A. E. Mirsky of the Rockefeller Institute for Medical Research; guanylic acid, sodium guanylate, and cytidylic acid were obtained from the Levene collection of chemicals at the Rockefeller Institute, also through the kindness of Dr. A. E. Mirsky. Photographic materials were provided from a grant to Columbia University by The Rockefeller Foundation.

Experimental Data

Intracellular occurrence and localization of alkaline phosphatase activity in the salivary gland cells of Drosophila melanogaster

Upon microscopic examination of smears of larval salivary glands of Drosophila, it is evident that in the preparations which have been incubated in the substrate, there is a much greater precipitate of cobaltous sulfide, indicating alkaline phosphatase activity, than in the smears incubated in control solutions. A diffuse precipitate occurs throughout the cytoplasm, but there is a much higher density within the nucleus (Fig. 1). This agrees with the observations of Moog (1944), on the tissues of the chick embryo that the nuclei were never less reactive than the cytoplasm and that the nuclei, unlike the cytoplasm, were never negative in reaction. Dounce (1943) using nuclei isolated from rat liver, also found that alkaline phosphatase activity is much greater in nuclei than in total liver.

Within the nucleus the precipitate is sharply localized in the chromosomes and nucleolus. The nuclei of the control slides show slight, if any, precipitate, with the exception that there is always some cobaltous sulfide in the control nucleoli (Fig. 2), but this is always considerably less in amount than that in nucleoli of substrate-incubated preparations.

The most dense precipitate is found within the chromosomes. When smeared in Ringer's solution, which is necessary to preserve the activity of the enzyme, the details of the chromosome structure are by no means as distinct as in the acetic acid smears that have been used in mapping bands. Nevertheless one can be certain that the cobaltous sulfide within the chromosome is concentrated in transverse bands, alternating with regions of very little or no precipitate (Figs. 3 and 5). This observation was reported earlier (Krugelis, 1945) and it has recently been confirmed by Danielli and Catcheside (1945) who compare the individual bands with specific regions of the cytogenetic chromosomal maps. In the preparations of Figures 3 and 5 the identity of the regions of phosphatase precipitate with the bands of acetic acid smears was established by a direct cytological observation. This method made use of the fact that the cobaltous sulfide precipitate is not altered by the standard Feulgen cytochemical procedure, that it becomes possible to superimpose the nucleal reaction on a phosphatase test. Figure 4 is from such a preparation; and comparison with Figure 5, the same chromosome after the phosphatase reaction but before the Feulgen test, shows that the bands in which the enzyme activity is localized correspond strictly with the Feulgen positive bands that form the basis of the familiar cytogenetic maps. The superposition of the second procedure merely changes the brown band to a purple brown, which is clearly a combination of cobaltous sulfide and basic fuchsin. This combination of the two cytochemical tests shows, in a vivid manner, that the highest level of alkaline phosphatase activity within the salivary gland cell occurs in exactly the region that contains a very high concentration of organic phosphate in the form of desoxyribose nucleic acid.

Properties and distribution of intracellular alkaline phosphatases

The occurrence of the enzyme activity and the desoxyribose nucleic acid within the same parts of the salivary gland chromosome suggests a functional relationship, which suggestion was put to test by using other phosphate esters as substrates for the enzyme activity. In order to elucidate a more specific picture of possible enzyme activity *in vivo* within the nucleus and the cell, esters were chosen which are normally present in the cell, and which the enzyme would consequently be expected to hydrolyze. The cellular substrates used, along with sodium glycerophosphate as the standard, were purine and pyrimidine nucleotides, ribose nucleic acid (presumably not highly polymerized), and desoxyribose nucleic acid (both polymerized and partially depolymerized). These substrates were tried on various mouse tissues for the presence of the enzyme activity in the cells. The testis was used for the chromosomal localization, since the other tissues contained nuclei which were mainly in the resting stages. In the results the black-brown precipitate of cobaltous sulfide indicates the site of alkaline phosphatase activity on the specific organic substrate in the incubating solution, and the density of the precipitate may be regarded as a measure of the intensity of the enzyme activity.

The photomicrographs (cf. Plates II, III, and IV) illustrate the results of these experiments; and roughly quantitative estimates of the intensities of the reaction are summarized in Table I. With sodium glycerophosphate and the nucleotides as substrates, the precipitate is not sharply localized, though it is always more dense in the nucleus and chromosomes than in the cytoplasm. By contrast, the reactions with depolymerized nucleic acids result in precipitates which are restricted to particular parts of the cell. On the slide in which depolymerized chromonucleic (desoxyribose nucleic) acid has been used as substrate the dark nuclei and chromosomes stand out very distinctly against a colorless cytoplasmic background, an appearance not unlike that of a Feulgen preparation. On the other hand, if plasmonucleic (ribose nucleic) acid is the substrate, the slide as a whole is intensely dark due to cytoplasmic precipitate, and the nuclei appear as light areas. The two types of slides compare with one another somewhat as do a photographic positive and negative. The chromonucleic acid precipitate is not uniformly distributed throughout the

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EXPLANATION OF PLATE I

The dark precipitate indicates the phosphatase activity. Photomicrographs made from smear preparations. Figures 1, 3–5 show phosphatase activity with sodium glycerophosphate as the substrate.

FIGURE 1. Alkaline phosphatase activity in salivary gland of *Drosophila melanogaster* larva. Magnification is 200 ×.

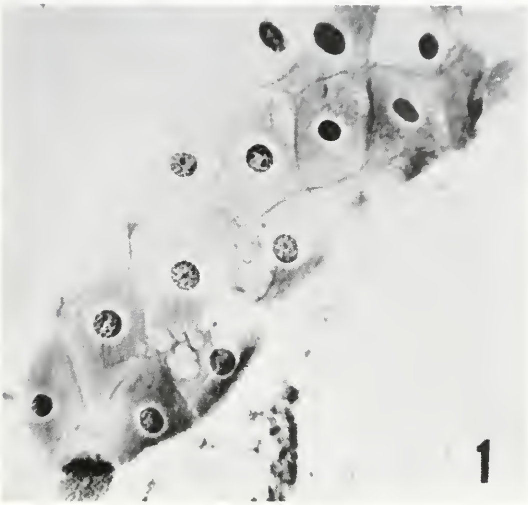
FIGURE 2. Salivary gland nucleus from control slide which had no substrate for the alkaline phosphatase activity. Magnification is 1000 ×.

FIGURE 3. Salivary gland nucleus showing alkaline phosphatase activity. Magnification is 1000 ×.

FIGURE 4. Salivary gland chromosomes with the Feulgen reaction applied over the phosphatase reaction precipitate. Magnification is 3000 ×.

FIGURE 5. Salivary gland chromosomes (same chromosomes as Fig. 4) showing alkaline phosphatase activity before Feulgen reaction applied over the precipitate. Magnification is 3000 ×.

PLATE I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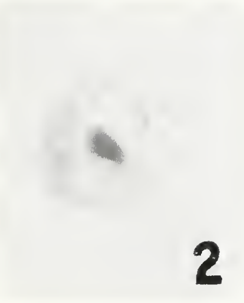
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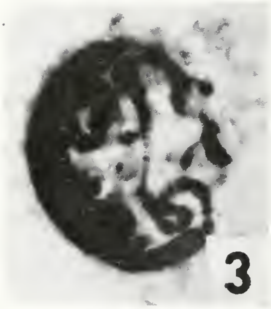
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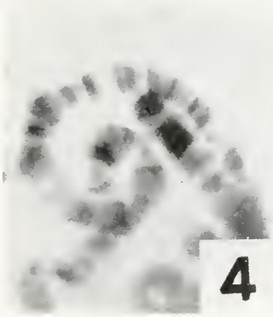
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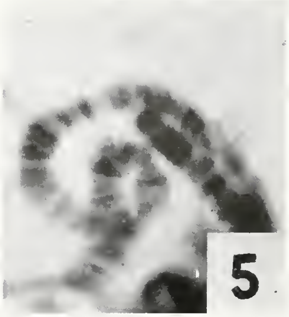
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nucleus. Instead it occurs only within the formed structures of the nucleus, the chromatin and the plasmosome nucleolus.

In an attempt to find enzyme differences involved in producing such specific localizations of alkaline phosphatase activity, these reactions were subjected to various conditions which might alter the reaction environment. Exposures to temperatures of 55° C. or over for a period of 5 minutes or longer produced complete irreversible inactivation of all the enzyme reactions. Magnesium ions in the concentration of 0.01 M magnesium sulfate showed some activation with the cytoplasmic reaction on ribose nucleic acid, and little or no increase on the nuclear and the general reactions. This very slight activating effect is in agreement with Schmidt and Thannhauser's (1943) observation that there is but slight effect of magnesium on intestinal alkaline phosphatase activity with sodium glycerophosphate as substrate.

TABLE I

Phosphatase reaction with different substrates

Substrate	Intestine		Testes		Liver		Pancreas		Kidney	
	c	n	c	n	c	n	c	n	c	n
Sodium glycerophosphate	1	2	1	2	0	2	±1	2	3	2
Adenylic acid, guanylic acid, cytidylic acid	1	2	1	2	0	2	0	2	1	2
Desoxyribose nucleic acid	0	0	0	0	0	0	0	0	0	0
Depolymerized desoxyribose nucleic acid	0	2	0	2	0	2	0	2	1	2
Ribose nucleic acid	2	±1	2	1	1	±1	2	±1	2	1

Density of cobaltous sulfide precipitate recorded from visual estimates. 0, no reaction; ± doubtful; 1, definite reaction; 2, strong reaction; 3, very strong reaction. Each record based on microscopic examination of at least 20 slides. Column c, cytoplasmic precipitate; column n, nuclear precipitate.

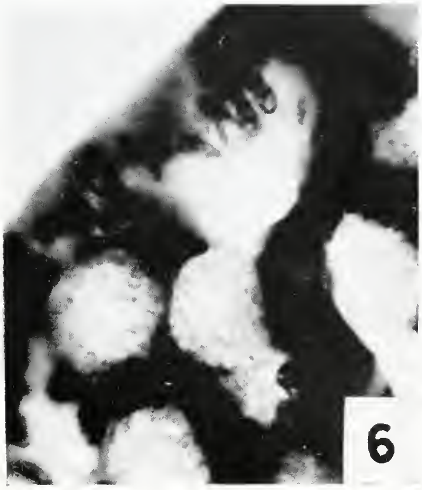
Cyanide ions in the concentration of 0.01 M potassium cyanide completely inhibited all three types of reaction.

Arsenate ions in the concentration of 0.01 M sodium arsenate showed a more selective effect on the different reactions than did the environmental influences mentioned above. In the presence of arsenate, the alkaline phosphatase activity with the nucleic acids as substrates is completely suppressed. The glycerophosphate splitting, by contrast, in most cases proceeds at its normal rate in a medium which contains arsenate. A roughly quantitative estimation of the inhibition or suppression of activity by arsenate ions is presented in Table II.

DISCUSSION

The differences in site of alkaline phosphatase activity in the same tissues when using different phosphate bearing substrates indicate localized reactions of three

PLATE II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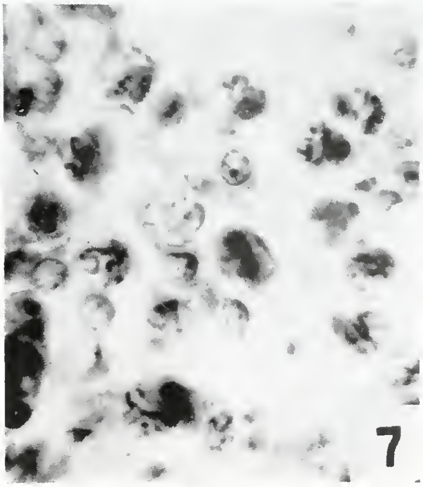
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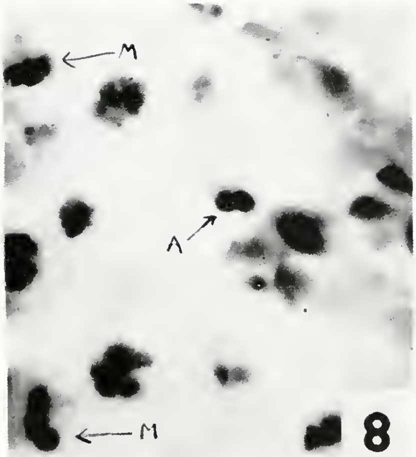

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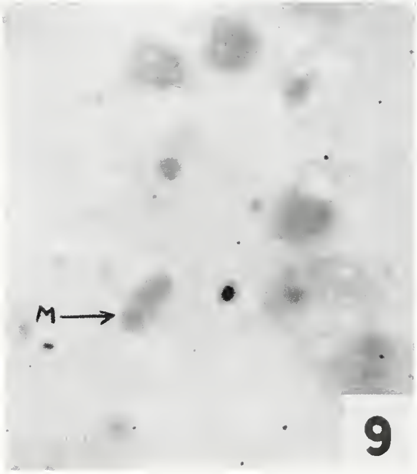

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Photomicrographs from 5 micra thick sections. Figures 6–8 show alkaline phosphatase activity with sodium glycerophosphate as substrate.

FIGURE 6. Small intestine of mouse showing activity of the enzyme distributed in the mucosa and submucosa layers. Magnification is 1000 ×.

FIGURE 7. Testis of mouse. Magnification is 1000 ×.

FIGURE 8. Testis of mouse. *M* indicates chromosomes in metaphase. *A* indicates chromosomes in anaphase. Magnification is 1000 ×.

FIGURE 9. Testis of mouse from a control slide with no substrate for enzyme activity applied. *M* indicates chromosomes in metaphase. Magnification is 1000 ×.

types, as follows: first, a general alkaline phosphatase reaction in both the cytoplasm and the nucleus, as in the case where sodium glycerophosphate and nucleotides are used as substrates; second, a definite nuclear reaction with little or no activity in the cytoplasm, as in the case of depolymerized desoxyribose nucleic acid as substrate; third, a definite cytoplasmic reaction with little reaction in the nucleus, as in the case of ribose nucleic acid as substrate. Since the enzymes cannot be isolated at this time, and since the nuclear and the cytoplasmic reactions are not absolutely specifically nuclear or cytoplasmic, they will be listed as reaction types with reference only to the location of the activity observed. With the different substrates used, the total reaction of phosphatase activity is somehow produced, and this is detected by the location of the cobaltous sulfide precipitate. The final reactions produced under a variety of substrate conditions must be due to at least three different enzymes, working in at least two different complexes.

TABLE II

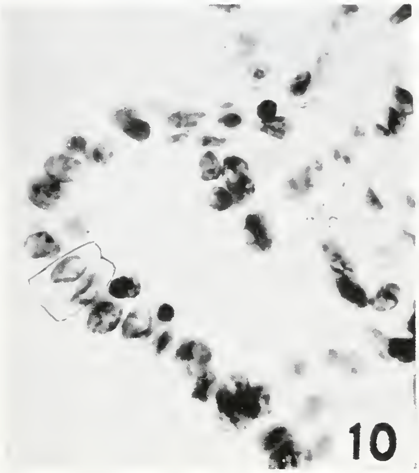
Intensity of precipitate of phosphatase reaction in different tissues using different substrates with and without arsenate ions

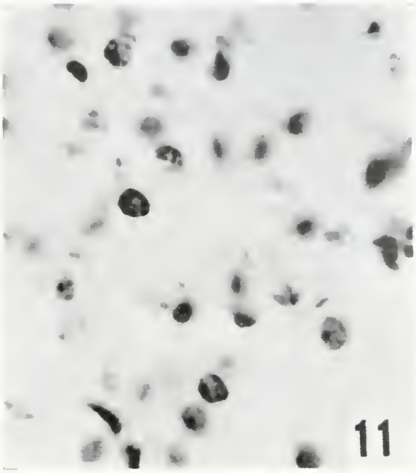
Substrate	Intestine		Testes		Liver		Pancreas		Kidney	
	c	n	c	n	c	n	c	n	c	n
Sodium glycerophosphate										
No arsenate ions	1	2	1	2	0	2	±1	2	3	2
With arsenate ions	1	1*S1	1	2	0	0*S3	±1	2*S0	2	±1*S1
Depolymerized desoxyribose n. acid										
No arsenate ions	0	2	0	2	0	2	0	2	1	2
With arsenate ions	0	0*S3	0	0*S3	0	0*S3	0	±1*S2	0	0*S3
Ribose nucleic acid										
No arsenate ions	2	±1	2	1	1	±1	2	±1	2	1
With arsenate ions	0	0*S3	2	0*S3	0	0*S3	0	0*S3	2	1*S0

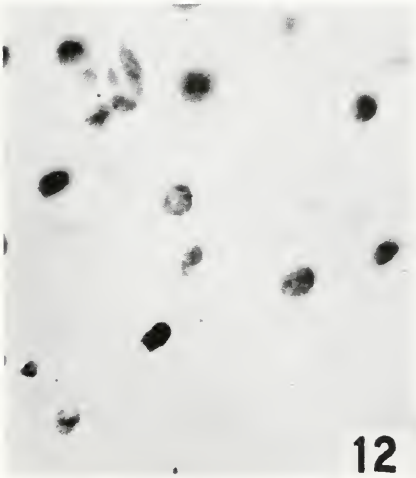
*S = suppression estimates.
S0 is no suppression.
S1 is slight suppression.
S2 is much suppression.
S3 is complete suppression.
Each record represents observation on 8 experimental slides.

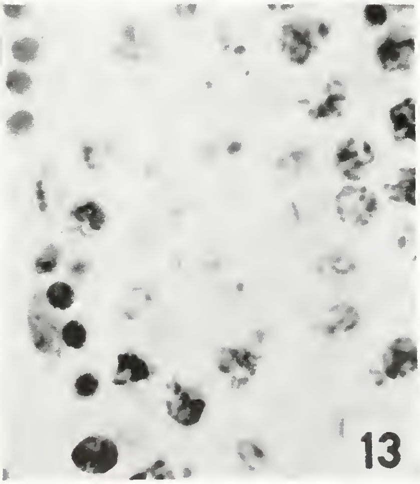
The suggestion that the reactions observed are due to complexes of enzymes is based on the chemical structure of nucleic acids. The ribose and desoxyribose nucleic acids, which were applied as substrates, are polymers of mononucleotides, which are considered to be linked to each other by an ester bond between the phosphate group of one nucleotide and the sugar group of the neighboring nucleotide, thus making the nucleic acids diesters of phosphoric acid all along the chain except in the terminal monoester of phosphoric acid. The total phosphatase action on the nucleic acids might be considered as essentially due to a specific desoxyribose nucleic acid phosphodiesterase, and a specific ribose nucleic acid phosphodiesterase action liberating mononucleotides of the nucleic acids, which then are hydrolyzed by a phospho-

PLATE III









Photomicrographs from 5 micra sections. Figures 10–13 show alkaline phosphatase activity with depolymerized desoxyribose nucleic acid as substrate.

FIGURE 10. Small intestine of mouse showing the distribution of the enzyme activity in the mucosa layer. Magnification is 1000 ×.

FIGURE 11. Kidney of mouse showing enzyme activity in the proximal tubules seen in cross-section. Magnification is 1000 ×.

FIGURE 12. Pancreas of mouse showing distribution of enzyme activity. Magnification is 1000 ×.

FIGURE 13. Testis of mouse showing enzyme activity. Magnification is 1000 ×.

monoesterase to liberate the inorganic phosphate. Thus the precipitate formed when the nucleic acids are applied as substrates is due to the activity of at least two different enzyme complexes, as follows: in the *cytoplasm*, first, a specific phosphodiesterase ("plasmonucleodiesterase") liberates ribonucleotides; second, phosphomonoesterase liberates inorganic phosphates from these nucleotides; in the *nucleus*, first, a specific phosphodiesterase[3] ("chromonucleodiesterase") liberates desoxyribosenucleotides; second, phosphomonesterase liberates inorganic phosphate from these nucleotides. This specificity of the diesterase follows from the fact that ribose nucleic acid will not serve as substrate for the nuclear diesterase activity, nor will desoxyribose nucleic acid serve for the cytoplasmic activity. With regard to the monoesterase activity, however, no such specificity has been detected, for the products of ribose nucleic acid hydrolysis serve equally well as substrates for phosphate production in either nucleus or cytoplasm.

While the action of the phosphodiesterase in freeing mononucleotides from lower polynucleotides (depolymerized nucleic acid) presumably can only occur by attack upon the linkage between the phosphate of one nucleotide and the sugar of the adjacent unit, it is evident that there is considerable restriction upon the exact nature of the bond which can be so attacked. If the enzyme were able to hydrolyze the bond at many points along a nucleic acid chain (consisting, let us say, of 2000 nucleotides) the diesterase should also function as a depolymerase, and a phosphate precipitate should be formed when the long, polynucleotide chains (polymerized nucleic acid) are used as substrate. Even after 72 hours action, however, there is no visible precipitate under these conditions, in contrast to the depolymerized nucleic acid experiments in which a dense precipitate is formed in a few hours. The type of bond which the diesterase can attack is evidently one which is enormously multiplied by a process of depolymerization. A most obvious view of enzyme specificity that would agree well with these facts is that the diesterase can attack only the bond between a terminal nucleotide and the penultimate nucleotide. Considering, for example, an extreme case, depolymerization of a 2000 unit polynucleotide chain to the minimum tetranucleotide should increase the number of bonds which such a specific diesterase can attack by a factor of 500. If specific terminal hydrolysis is the mechanism, the amount of mononucleotide that would become available by diesterase action upon the end of a highly polymerized nucleic acid chain would surely give an amount of phosphate precipitate so slight that it would be cytologically undetectable.

It is highly important to the question of the functional significance of the distribution of the phosphatases acting on the nucleic acids, that this actually coincides in a striking manner with the known locations of desoxyribose and ribose nucleic acids within the cell. The phosphatase activity on depolymerized desoxyribose nucleic acid is restricted to the chromatin of the nucleus, the only part of the cell in which this type of nucleic acid is found. By contrast, the ribose nucleic acid phosphatase activity is in the cytoplasm, a region in which only ribose nucleic acid has ever been demonstrated. (The only marked discrepancy is the occurrence of desoxyribose

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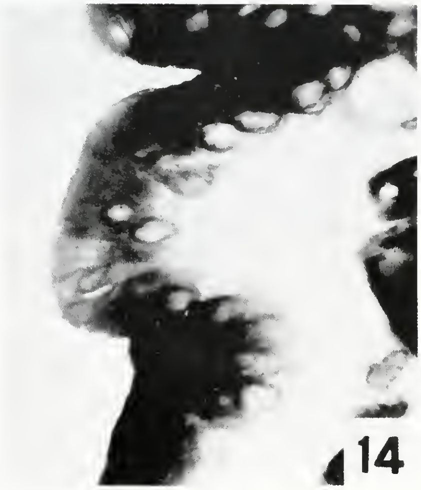
[3] Concerning the existence of this nuclear diesterase, there is some possible supporting evidence from the work by Mazia and Ballentine, reported by Mazia (1941), on an intranuclear enzyme from Arbacia eggs. Their enzyme, termed polynucleotidase, was active at a pH 9.0 and was capable of reacting on desoxyribose nucleic acid still in a polymerized form.

Plate IV



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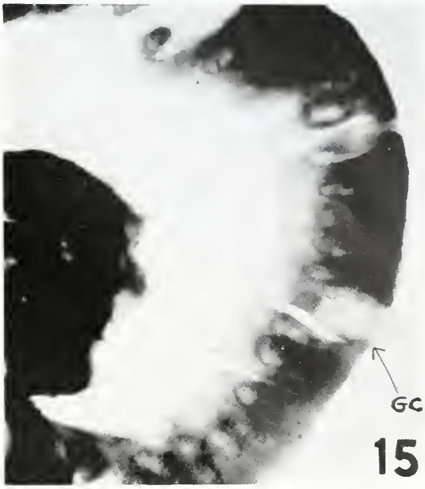

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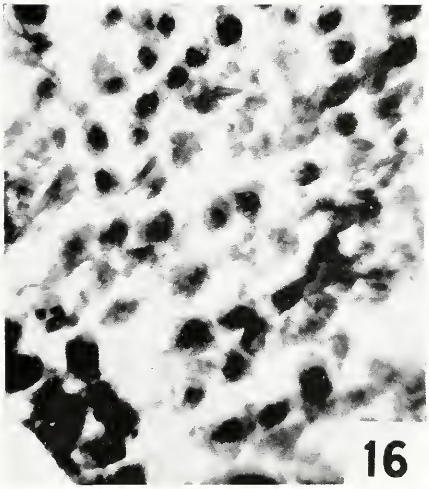

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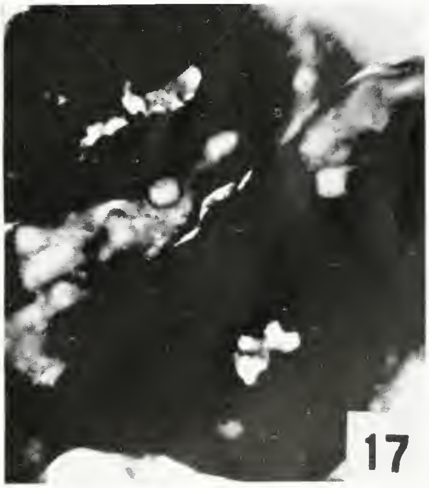

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Photomicrographs from 5 micra thick sections. Figures 14–17 show alkaline phosphatase activity with ribose nucleic acid as the substrate.

Figure 14. Small intestine of mouse showing the enzyme activity in the cells of the mucosa layer. Magnification is 1000 ×.

Figure 15. Small intestine of mouse showing goblet cells in the mucosa layer and the distribution of the enzyme activity. Magnification is 1000 ×.

Figure 16. Testis of mouse showing the enzyme activity. A denser precipitate is found in the nuclei of testis cells than in nuclei of other tissues under the conditions of the same substrate. Magnification is 1000 ×.

Figure 17. Kidney of mouse, showing enzyme activity in the proximal tubules in cross-section. Magnification is 1000 ×.

nucleic acid phosphatase activity in the nucleolus, which, since it is Feulgen negative, is considered to contain ribose nucleic acid.)[1]

Certain possible *in vivo* functions of these alkaline phosphatases are at once obvious. Not only can the nuclear diesterase split off terminal mononucleotides, as in these experiments, but in the reverse direction, it may conceivably catalyze the terminal growth in the development of nucleotide chains. One may easily picture the later stages of synthesis of a full length nucleic acid chain as involving the cooperation of two enzymes: the diesterase slowly builds up short chains by terminal growth and this is followed by the action of the depolymerase type of enzyme catalyzing the union of these short chains into the long complex which is such an important structural component of a chromosome.

While it is also obvious that catalysis of the synthesis of a mononucleotide from a nucleoside by phosphomonesterase is an essential step in nucleic acid synthesis, one's attention here tends rather to focus on the possibilities of dephosphorylation of nucleotide as a source of energy for nuclear and cytoplasmic reactions. Thus energy for synthesis of chromosomes and their products may or may not be available according to whether the nucleotides are structurally isolated from phosphomonesterase activity by being bound in nucleic acid chains, or whether as a result of a successive action of nuclear depolymerase, and "chromonucleodiesterase," there is mononucleotide available for dephosphorylation. Similarly the actual availability of energy for such cyclic nuclear mechanical processes as chromosome coiling and mitotic movement may be dependent upon a cycle of binding and release of mononucleotide from its nucleic acid storehouse.

SUMMARY

1. Using the histochemical test for alkaline phosphatase reaction in the larval salivary glands of several species of Drosophila, activity was found to be present in three main parts of the cell; the cytoplasm, the nucleolus, and the chromosomes.

2. Phosphatase activity was found rather generally distributed in both the cytoplasm and the nucleus. Within the larval salivary gland chromosomes, the enzyme activity was localized in those chromosomal regions which are Feulgen positive, and thus corresponds to the regions containing large concentrations of desoxyribose nucleic acid.

3. Different naturally occurring phosphate bearing substances were used as phosphatase substrates on mouse tissues, and resulted in demonstration of three different types of phosphatase reactions based on the localization of the enzyme activity.

a) A general reaction with phosphatase activity located in both nucleus (nucleolus and chromosomes) and the cytoplasm was present when sodium glycerophosphate and nucleotides were used as substrates.

b) No phosphatase reaction occurred on polymerized desoxyribose nucleic acid, but a specific nuclear reaction (nucleolus and chromosomes) was present when nuclease-depolymerized desoxyribose nucleic acid was used as a substrate.

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[1] We do not actually know the location of the possible substrates for the diesterase activity, since the methods for localizing nucleic acids do not, in all likelihood, preserve any but the high polymers, and it may well be that the lower polymers of the sort used as substrates are not the same in distribution as the larger complexes. In such a difference may lie the explanation of the discrepancies above.

c) A strong cytoplasmic reaction with slight reaction in the nucleus was present when ribose nucleic acid was used as a substrate.

4. Subjection to several environmental variables produced little further evidence as to the differences among these three types of localized reactions.

5. The three types of alkaline phosphatase reactions observed were suggested to be due to at least two phosphodiesterases and a phosphomonesterase.

6. The nuclear phosphatase complex and the cytoplasmic phosphatase complex each probably consist of a specific phosphodiesterase, which splits the ester linkage between the phosphate of one nucleotide and the sugar of the neighboring nucleotide, and a phosphomonesterase which splits the second ester linkage and liberates inorganic phosphate.

Acknowledgment

I wish to express my appreciation and gratitude to Professor H. Burr Steinbach for originally bringing to my attention the cytochemical possibilities of the alkaline phosphatase test; to Professor Arthur W. Pollister for his advice and encouragement throughout the course of the work and for his help in the preparation of the manuscript; to Dr. Alfred E. Mirsky for advice and essential materials; and to Dr. Robert Ballentine and Dr. Jack Schultz for many helpful suggestions.

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PHYSIOLOGY OF INSECT DIAPAUSE: THE ROLE OF THE BRAIN IN THE PRODUCTION AND TERMINATION OF PUPAL DORMANCY IN THE GIANT SILKWORM, PLATYSAMIA CECROPIA

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The phenomenon of insect diapause presents an exceptionally clear statement of one of the most important problems in biology; to wit, the nature of the factors that preside over cellular growth and differentiation. For with the onset of diapause and through the workings of internal physiological mechanisms still to be elucidated, growth suddenly comes to a standstill and the animal for months thereafter persists in a genuine state of suspended development. With the termination of diapause the rapid tempo of cellular activity returns and metamorphosis continues where it had left off. Thus whatever may be the inner mechanism for the induction and termination of diapause, it must have the capacity to turn morphogenesis off and on in a most striking way.

The study of this phenomenon has not failed to claim the attention of a large array of investigators. For example, even in 1932, Cousin was able to cite 347 papers in a review of the literature. That this extensive literature has so imperfectly advanced our knowledge of diapause is apparently due to the fact that most investigations have been carried out either on muscoid flies, which have a most imperfect and complex diapause, or on the eggs of silkworms and grasshoppers, which are too small to permit extensive manipulations of the individual animal. In the present investigation these difficulties were minimized by working on species of insects that possess a wholly characteristic pupal diapause, and, by virtue of weighing up to 8 grams per individual, are among the very largest insects in America.

MATERIALS AND METHODS

Pupae of the giant silkworm, *Platysamia cecropia,* were used for the most part, approximately 1200 pupae being studied in a total of 690 experiments. These insects were reared from eggs obtained from fertile females; a lesser number of pupae were secured from dealers. In my experience, this species has never failed to enter into diapause immediately after pupation, thus giving only one brood a year. If the pupae are maintained constantly at room temperature, diapause persists for not less than five months; if they are placed immediately after pupation at a temperature of 3° to 5° C. and chilled for 1½ months or longer, adult moths emerge about 1 to 1½ months after being returned to room temperature. For this reason the stock of material was divided at the outset into two batches, one being placed and stored at 3° to 5° C. until needed ("chilled pupae"), and the other being maintained at room temperature where, as previously described, diapause persists for at least five months ("diapausing pupae").

In a number of experiments related species of saturniid pupae were used; namely, *Samia walkeri*, *Callosamia promethea*, and *Telea polyphemus*.

The most important factor facilitating the investigation was the discovery of a method of continuous anesthesia for insects during operative procedures. This method, utilizing carbon dioxide and described by Williams (1946), permitted extensive and prolonged surgical manipulations without any loss of blood or apparent damage to the pupae. Other procedures will be described as encountered in the following discussion.

PARABIOTIC EXPERIMENTS

We have noted that diapausing pupae, after a period of exposure to low temperature, are rendered competent to develop when returned to room temperature, whereas, in contrast, pupae not subjected to chilling remain in diapause for at least five months. With these two types of animals at hand one is therefore in a position to test the fundamental nature of diapause by simply grafting one to the other so that they share the same blood. If diapause results from the *presence* of some factor inhibiting development, then such parabiotic combinations should fail to develop by virtue of the diapausing pupa distributing this inhibitory factor to the chilled individual. To the contrary, if diapause results from the *absence* of some necessary growth factor, both animals should develop, provided the chilled individual can supply double the minimal amount needed by a single animal.

In making these preparations a disc of pupal cuticle plus underlying hypodermis was cut from each pupa and the two animals placed together and held thus by the application of melted paraffin around the site. Most of the pupae were joined at the thoracic tergum (Figs. 1 and 3), but occasionally the junction was accomplished at the head or at the tip of the abdomen. Provided that the underlying heart is not injured and that no bubbles of air are trapped in either animal, such combinations are easily established and a high percentage survive.

In order to demonstrate that the operation in itself is without effects on dormancy, a series of ten diapausing pupae were successfully grafted to diapausing partners. Diapause persisted in each of these animals, adult moths being produced only after a minimum of 5½ months, the usual minimum length of time necessary for the spontaneous termination of diapause at 25° C. To the contrary, when diapausing pupae were joined to previously chilled individuals, the diapause in all viable preparations was terminated. In a series of 15 such combinations the pairs emerged as fully formed, active moths in an average of 41 days. Metamorphosis was complete both externally and internally, the only defect being a failure of the wings to expand after emergence (Fig. 2). This activation was not species- or, indeed, genus-specific, for it was possible to terminate the diapause of *Platysamia cecropia* by joining them to previously chilled pupae of *Telea polyphemus* (Figs. 3 and 4). Furthermore, sexual differences were without significance, for male pupae had the capacity to induce development of females, and vice versa.

A striking feature of all these parabiotic combinations is the fact that the animals invariably grow together so as to be connected by a pedicle, a phenomenon first noted by Crampton (1899) and later by Wigglesworth (1936) and Bodenstein (1938) in grafting procedures on insects. We shall have occasion subsequently to consider this union more fully, but in the present analysis suffice it to say that the epithelial pedicle becomes externally chitinized and by way of its lumen permits a circulation of blood between the two animals.

In the earlier preparations the blood of the diapausing and chilled pupae in parabiosis was daily propelled to and fro by pressing accordion-like on the abdomen of each pupa alternately. This was later found to be wholly unnecessary, since development begins just as promptly without such forced mixing. It may also be noted that the completion of adult formation in the previously chilled animal occurs about 1½ days earlier than in the diapausing partner (Fig. 5). This results from a corresponding delay in the initiation of development of the diapausing pupa. At all stages in adult differentiation the chilled pupa is therefore approximately 1½ days in advance of the diapausing partner.

Thus these initial parabiotic experiments indicate some of the essential features of diapause. In general, they support the proposition that diapause results from the absence of a non-species-specific growth factor that is able to pass in parabiotic preparations from the activated to the dormant individual and evoke the initiation of adult development in the latter also.

Brain Implantation into Diapausing Pupae

If the termination of diapause is, indeed, accomplished by the action within the previously chilled pupa of a factor necessary for adult development, then it should be possible to demonstrate the organ in which this factor arises. For this purpose, various tissues and organs were removed from chilled pupae and implanted singly into diapausing individuals. When experiments of this sort were carried out, it was found that only one organ in the chilled pupa has the power to evoke development of diapausing pupae and that this organ is the brain itself. When the brain is removed from a chilled pupa and implanted into the head, thorax, or abdomen of a diapausing pupa, the latter is invariably induced to undergo adult development. Furthermore, *Platysamia cecropia* can be activated by the brains of *Samia walkeri, Callosamia promethea*, or *Telea polyphemus*—and, in fact, as far as the termination of diapause is concerned, there is a lack of species- and genus-specificity of brains among all these Lepidoptera tested. No other organ in the chilled pupa apparently possesses this power.

This effect of chilled brains is in marked contrast to that of diapausing brains,

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Explanation of Plate I

Approximately Life Size

Figure 1. Brainless, diapausing pupa of *P. cecropia* grafted to a chilled pupa of the same species.

Figure 2. Animals in Figure 1, after adult formation. The two insects have grown together and developed essentially simultaneously.

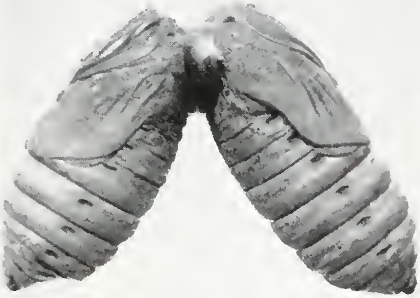
Figure 3. Brainless, diapausing pupa of *T. polyphemus* grafted to a chilled pupa of *P. cecropia*.

Figure 4. Animals in Figure 3, after adult development.

Figure 5. Parabiosis between two pupae of *P. cecropia*, removed from pupal cuticle before the completion of adult development. Development of the chilled pupa is 1½ days in advance of that of the brainless, diapausing pupa.

Figure 6. Adult Cecropia moth produced by a brainless, dipausing Cecropia pupa, whose diapause was terminated by implantation of a brain from a chilled Polyphemus pupa.

PLATE I



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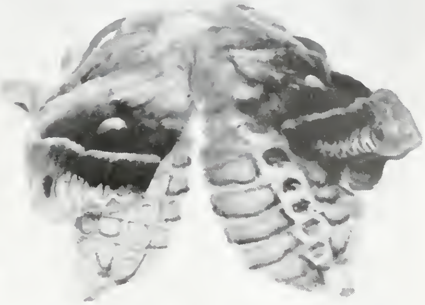

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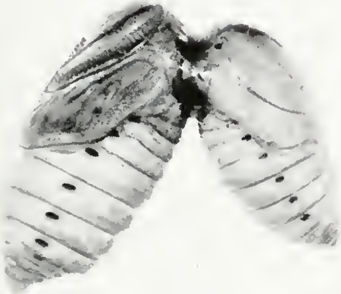

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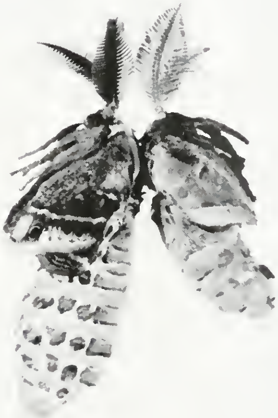

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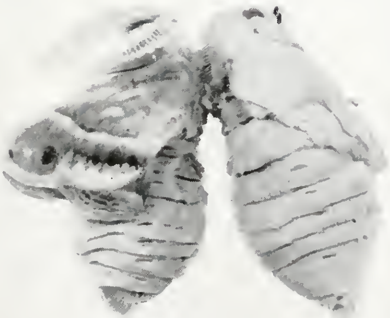

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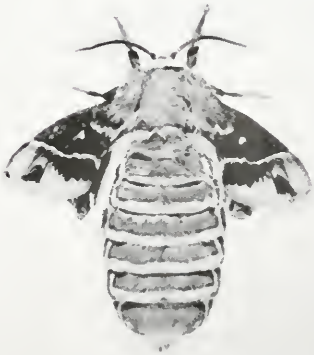

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for the latter fail to terminate dormancy even when as many as eight are implanted into a single pupa.

The lack of species-specificity of implanted, chilled brains suggested the possibility that the effect might, conceivably, be mediated by an activation of the endogenous brain of the diapausing, "host" pupa itself. For this reason the vast majority of subsequent experiments were carried out on diapausing pupae from which the brains had been removed.

Removal of Brain

The operation devised for this purpose is easily performed, as follows. The insect, anesthetized with carbon dioxide, is placed on its back and by means of a sharp scalpel a rectangular window of pupal cuticle excised from its face. The underlying, semi-transparent hypodermis is thus exposed. With microscissors the latter is trimmed away, along with the tracheae traversing the operative field. In this procedure the frontal ganglion is frequently removed, but this has been found to be inconsequential. The brain now lies exposed and can be floated up toward the operator by pressing the pupal abdomen so that further dissection is performed in the pupal blood. With finely ground jeweler's forceps the nerves passing laterally to the site of the future adult eyes are grasped and broken. In similar fashion each brain hemisphere is carefully broken loose, in turn, from its tracheal supply, from the nerve passing posteriorly to the corpus allatum and corpus cardiacum and from the circumesophageal connective. The brain can then be lifted free and examined in insect Ringer's solution.[1] Taking care to exclude all bubbles, the defect in the pupal chitin is then capped over with a small rectangle of thin, transparent plastic (cut from a plastic cover slip), which is sealed in place with melted paraffin.

The operation was originally performed with due regard to surgical asepsis; this was later found to be of little importance since the pupae are apparently not affected by the usual contaminating organisms. The mortality from the procedure is low and one ends up with a brainless pupa possessing a transparent window at its anterior end.

The defect in the hypodermis is rapidly repaired by a deposit of blood cells, followed by an ingrowth of cells and of tracheoles along the under surface of the plastic slip. Simultaneously, an intervening, delicate, transparent, "chitinous" lamella is elaborated. These relations permit a detailed study of the behavior of the hypodermis beneath the window, and, by means of an Ultropaque, cellular activity has been followed under the oil immersion objective. It may be noted that this local process of repair occurs just as promptly in diapausing as in previously chilled pupae. Notwithstanding this fact, the process of repair is without overall effects on dormancy.

Brain Implantation into Brainless Diapausing Pupae

The removal of the brain from diapausing pupae prior to using the animals experimentally proved to be an exceptionally significant maneuver. For whereas, as we have previously noted, diapausing pupae kept at 25° C. begin to escape spontaneously from diapause after about five months, no such activation occurs if the brain is removed. Among approximately 400 such pupae there has not been a single case of spontaneous development. It is therefore apparent that by removing the brain the pupa is maintained in permanent diapause. Such pupae remain alive for up to a year and finally die of dessication. Yet at any time during this period the diapause can be terminated by implanting into the brainless pupa the brain of a previously chilled animal (Fig. 6). Data in regard to a series of such pupae are given in Table I.

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[1] This physiological solution was originally devised by Ephrussi and Beadle (1936) for studies of Drosophila, but it works equally well for the Lepidoptera used in the present experiments. I am indebted to Dietrich Bodenstein for calling my attention to its composition, which is as follows: NaCl, 7.5 gm.; KCl, 0.35 gm.; and $CaCl_2$, 0.21 gm., per liter of water.

TABLE I

Evocation of Adult Development of Brainless Diapausing Pupae by Implantation of Brains from Chilled Pupae

Species of host	Species of implanted brain	Number of experiments	Aver. time for adult emergence
P. cecropia	*P. cecropia*	16	35 days
P. cecropia	*T. polyphemus*	2	89
P. cecropia	*S. walkeri*	3	72
P. cecropia	*C. promethea*	2	63
T. polyphemus	*T. polyphemus*	2	64
T. polyphemus	*S. walkeri*	2	28

Manifestly, these experiments demonstrate that the termination of diapause requires the presence of an activated brain, in the absence of which adult development of these insects has not been observed. The conclusion is also self-evident that the termination of dormancy after diapausing pupae have been chilled results from the action of low temperature in rendering the brain able to evoke adult development. The other tissues in the diapausing pupa do not require such exposure to cold, for they are rapidly activated by implanting a brain which, alone, has been chilled.

This fact can also be readily demonstrated by removing strips of integument from diapausing pupae and implanting them into previously chilled pupae. Such diapausing tissues develop simultaneously with the host: the pupal cuticle is delaminated and a normally chitinized, adult cuticle, complete with scales and hairs, is found in the implant, in exactly the same fashion as described by Piepho (1938a and b) and Kühn and Piepho (1940) in studies of other aspects of insect metamorphosis. Thus the effect of low temperatures in facilitating escape from diapause can be explained solely on the basis of its effect on the brain.

PARABIOTIC EXPERIMENTS ON BRAINLESS DIAPAUSING PUPAE

As soon as the brain was definitely shown to be the source of the factor terminating diapause, ten more parabiotic combinations were prepared, but this time uniting *brainless* diapausing pupae with chilled individuals. Identical results were obtained: the two pupae in each combination grew together by a chitinized, epithelial, blood-filled pedicle and after an average of 44 days emerged as normal, active, adult moths.

BRAIN REMOVAL FROM PREVIOUSLY CHILLED PUPAE

Further information concerning the action of the brain in terminating diapause can be gained from a consideration of the behavior of chilled pupae. We have previously noted that these animals undergo no apparent development as long as they are maintained at the low temperature, but that within 1 to 1½ months after return to 25° C. the adult moth has fully formed and emerges. It is therefore worthy of note that if the brain of such chilled pupae is removed as soon as the insect is returned to the warm temperature, adult development never occurs and, in the same fashion as described for brainless diapausing pupae, dormancy persists indefinitely until the animal finally dies of dessication. Yet development can at any time be evoked by merely implanting into the head, thorax, or abdomen a brain obtained from another chilled pupa.

This phenomenon was studied more fully as follows. A series of thirty previously chilled pupae was placed at 25° C. and every few days the brains from several of these insects were removed and implanted into brainless diapausing pupae. The results may be summarized most briefly by saying that when the brain is removed within approximately the first 11 days, the brainless donors never show any development; such brains, in turn, evoke the development of brainless, diapausing pupae. In contrast, if the brain is removed from previously chilled pupae after approximately 17 days at 25° C., development continues to produce normal, brainless adults and the removed brains are without effect in terminating the dormancy of brainless, diapausing pupae.

These experiments have been repeated on a large scale, with special attention to the effect of brain removal during the critical period of 11 to 17 days. These more detailed studies were facilitated by establishing, at the outset, a transparent, plastic, facial window in each chilled pupa so that the behavior of the underlying hypodermis could be observed. It was at once apparent that the critical period, 11 to 17 days, was, in a sense, a statistical artifact, since, during this period, each individual achieves threshold activation during an extremely short interval, not exceeding a few hours. The actual critical period for each pupa is signaled by the initiation of hypodermal retraction from the overlying, facial chitin. Prior to this point, removal of the brain prevents development, and such brains evoke the development of brainless, diapausing pupae after an additional latent period of approximately three weeks. The moment hypodermal retraction is initiated, the brain can be dispensed with and such brains, when tested, are inactive.

Thus it is apparent that diapause persists even in chilled pupae until the latter have been exposed to a developmental temperature for an average of two weeks. Consequently, the activation of the pupal brain during exposure to low temperature must be conceived in terms of some physical or chemical alteration in the brain substance whereby the latter is rendered competent to produce or release its stimulating factor during subsequent exposure to a developmental temperature The brain's action is then exerted and, thereafter, metamorphosis can proceed independent of its further participation.

Role of the Corpora Allata

All the evidence so far considered reveals the brain as the organ of paramount importance in engendering and terminating diapause. Thus diapause in these species appears to result from the absence of a factor necessary for adult development, rather than from the presence of an inhibitory factor. The possibility remained, however, that the failure of the brain to exert its effect and the consequent onset of diapause might, in turn, be due to inhibition arising elsewhere in the organism. The corpora allata were deemed the most likely source of such hypothetical inhibition and for this reason their significance in the production of diapause was studied.

As originally demonstrated by Bounhiol (1938) and subsequently confirmed by Piepho (1940; 1941), the corpora allata of Lepidoptera specifically inhibit pupation during the larval instars and thus oppose the activation of the presumptive imaginal tissues. This finding seemed so significant that comparable experiments were carried out on the caterpillars of *Platysamia cecropia* and *Telea polyphemus*. Although the removal of the corpora allata from caterpillars is a difficult procedure, it was accomplished in a sufficient number of immature (fourth instar) larvae to demonstrate that precocious pupation, indeed, results therefrom, the usual fifth instar being omitted. Furthermore, there is convincing evidence that the function of the corpora allata in inhibiting the imaginal discs disappears during the final larval instar and pupation then ensues. These findings have been considered in some detail for, although they concern pupation rather than adult differentiation, it is easy to see the importance of demonstrating whether, in potentially diapausing insects, the corpora allata once again inhibit the imaginal tissues, or the brain, and thus participate in the induction of diapause.

A series of experiments was therefore performed in which the corpora allata were removed (by a frontal approach) from diapausing pupae. Such pupae[2] invariably continued to diapause normally, and the removed corpora allata when implanted into previously chilled pupae were without effect in inhibiting adult development. As many as six corpora allata have been implanted into a single chilled pupa without retarding metamorphosis.

Similar negative results were obtained in regard to all other organs studied as a possible source of some inhibitory factor. For example, the diapausing brain itself is without inhibitory properties, for as many as six such brains have been implanted into a single chilled pupa without producing diapause. This is also true for the subesophageal ganglion, thoracic ganglion, gonads, imaginal discs, and strips of integument.

Although it cannot be denied that inhibitory factors may play a role in the production of diapause, the sum total of available evidence offers nothing to support this proposition. The brain itself remains the key to the production and termination of diapause in the species studied.

Discussion

The role of the brain in terminating diapause, demonstrated for the first time in the present investigation, can to advantage be compared with its other functions in insect metamorphosis. Thus, in the bug, Rhodnius, the brain is necessary for moulting (Wigglesworth, 1940) and in the Lepidoptera it is now well established that the brain is also required for pupation (Kopec, 1922; Caspari and Plagge, 1935;

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[2] It may be noted that the allatadectomized pupae ultimately escaped from diapause after the usual minimum period of 5½ months at 25° C. The resulting moths were wholly normal in all respects and could be induced to mate and lay eggs, which, in turn, were fertile. These findings apparently deny a participation of the corpora allata in the egg production of these species, a function described for them in certain other Orders of insects (Wigglesworth, 1936; Pfeiffer, 1939).

Kühn and Piepho, 1936; Bounhiol, 1938; Piepho, 1938a; Plagge, 1938). A surprising fact is that a role of the brain in imaginal differentiation has been specifically denied by all of these investigators of lepidopteran metamorphosis. The important point is that this conclusion was, without exception, based on studies of continuous, non-diapausing development. For such insects there can be little doubt that adult formation ensues even though the brain is removed from mature caterpillars in the last instar (i.e., after the "critical period" for pupation). The existence of this striking difference between continuous and diapausing development has been pointed out previously (Williams, 1942).

In the present investigation we have seen that all the evidence supports the theory that diapause results from an interruption in the normal processes of adult development. This point of view suggests that the brain is also necessary for evoking adult development in non-diapausing pupae. In non-diapausing individuals the brain may be viewed as having achieved its full developmental function precociously prior to pupation, whereas, in potentially diapausing animals, the brain first controls pupation and then months later after pupation it controls adult formation.

The action of the brain in terminating diapause in these saturniid pupae poses an additional problem of even greater interest; namely, the nature of the factor arising in the brain which so spectacularly evokes in dormant tissues a veritable flood of cellular activity. This problem will be considered in a subsequent communication.

SUMMARY

1. The physiological control of pupal diapause has been studied on a total of approximately 1200 pupae of the giant silkworms, *Platysamia cecropia*, *Telea polyphemus*, *Samia walkeri*, and *Callosamia promethea*.

2. The dormancy of diapausing pupae can be terminated readily by grafting them to activated (previously chilled) pupae. The two animals in each parabiotic combination grow together and some factor necessary for adult development passes from the activated to the dormant animal so that both develop simultaneously. This factor is not species- or genus-specific.

3. By implantation experiments the source of the factor terminating diapause is shown to be the brain and in this function a lack of species- and genus-specificity of brains is demonstrated.

4. In these species the well-known action of low temperatures in facilitating escape from diapause results from the effect of cold in rendering the brain competent to terminate domancy. Actual termination of dormancy is accomplished only after the previously chilled brain has been exposed to a developmental temperature for an average of two weeks. The earliest indications of adult development then become evident and the brain, thereafter, is no longer required for the completion of metamorphosis.

5. Therefore, the effect of low temperatures on the brain must consist in some physical or chemical alteration in its substance whereby the latter is rendered competent to produce or release an imaginal-differentiation factor after return to a developmental temperature.

6. No evidence was found to support the theory that diapause results from the

presence of inhibitory factors. In this regard, the functions of the corpora allata are considered in some detail.

7. It is concluded that diapause in these species results from an interruption in the normal processes of development by virtue of a failure of the brain to supply a non-species-specific factor necessary for adult differentiation. Diapause is terminated when this factor is provided.

8. The significance of the brain in the development of diapausing pupae is considered in relation to its other functions, as reported in the literature. Notwithstanding a certain amount of evidence to the contrary, it is probable that even in the absence of diapause the brain plays a vital role in adult formation.

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FEEDING OF OYSTERS IN RELATION TO TIDAL STAGES AND TO PERIODS OF LIGHT AND DARKNESS

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INTRODUCTION

The beds of the American oyster, *Ostrea virginica*, are usually situated in inshore areas where tidal currents are strong and their regularity is sharply defined. Because of an almost continuous flow of water generated by the tidal movements over the beds populated with oysters, these mollusks live under continuously changing conditions. There is no doubt that the environmental changes caused by the tides may in certain instances noticeably affect the behavior of the oysters. It has never been satisfactorily demonstrated, however, that some of the vital processes of oysters, such as feeding, are carried on more vigorously during certain stages of the tide.

A review of the literature on this subject shows that the only attempt to study the relation between the feeding activities of oysters and the stages of the tide was made by Nelson (1921) who came to the conclusion that "The times of complete cessation or of commencement of feeding shows a rather definite correlation with the stage of the tide." In his other article on the feeding habits of oysters Nelson (1923) states that in the case of these mollusks relatively little food is taken on the ebb tide. Even as late as 1938 Nelson still refers to his old observations emphasizing the relative inactivity of the oyster during the outgoing tide (Nelson, 1938). These conclusions were accepted without verification by some other investigators and were finally incorporated in the article on oysters in the Encyclopaedia Britannica where it is stated that "The American oyster does not feed late at night and in early morning, and relatively little on the ebb tide" (Orton, 1929). At present, therefore, such behavior of oysters is recognized as an established fact.

In our investigations of various aspects of the biology of oysters of Long Island Sound and its tributaries, especially of Milford Harbor, it was noticed that these mollusks fed actively on the ebb tide and also during darkness. The stomachs of the oysters removed from the water during such periods contained large quantities of food. It was also observed on numerous occasions in the day time that during the last stages of the ebb the oysters populating the shallow flats were wide open and apparently feeding because they were expelling true faeces. These observations suggested that the conclusions expressed by Nelson and Orton probably did not apply to the oysters of the Long Island Sound and Milford Harbor region. However, since our observations were only of an occasional nature, they could not be regarded as entirely reliable. Therefore, to ascertain the feeding habits of our oysters in relation to the tidal stages, and to the periods of light and darkness, a systematic series of experiments was conducted in the Summer and Autumn of 1945. The solution of the problem undertaken was approached by using several methods, each of which contributed toward the correct evaluation of the results of the others.

The authors wish to express their sincere appreciation and thanks to Miss Frances Tommers for her helpful participation in all the phases of this work and especially for the difficult analysis of numerous kymograph records obtained in the course of these studies.

OBSERVATIONS AND EXPERIMENTS

Stomach content

The first method consisted in examining the volume of the stomach content of the oysters collected during different stages of the incoming and outgoing tides. As a rule, the oysters examined were approximately 4 inches in length. The examination was made immediately after the specimens were taken out of the water. After opening an oyster an incision penetrating to the stomach was made through the body wall and the content of the stomach was collected by means of a fine pipette inserted through the incision.

At first it was intended to employ a volumetric method for determining the quantities of food found in the stomachs but, eventually, this method was found unsatisfactory because the bodies of the oysters, regardless of quite a uniformity of their shells, displayed considerable difference in their volume and size. Since small-bodied oysters possessed smaller stomachs and were probably incapable of ingesting quantities of food equal to those of larger individuals, serious errors could be introduced in the interpretation of the results. Therefore, another method, in which each oyster was considered individually, was decided upon. This method consisted in a direct evaluation of the relative quantity of food found in the oyster's stomach. Three classes, namely: "large," "small," and "absent," were established to designate the results of the examination. The oysters which were classified as containing large quantities of food could easily be recognized because, as soon as the incision was made in their bodies, the stomach content freely flowed out. This phenomenon is probably familiar to all investigators who worked with oysters. The animals placed in the second class were those whose stomachs contained only small quantities of food. The third class was composed of oysters with empty stomachs. The animals of the latter group were especially carefully examined to be certain that they contained no food.

The largest number of oysters examined in the course of these studies was collected from the cultivated oyster beds of Long Island Sound. This group, consisting of 1000 individuals, was composed of ten samples, each usually containing 100 oysters. These samples were collected at approximately weekly intervals during June, July, and August 1945. Each of the large samples consisted in turn of ten smaller ones composed of 8 to 12 individuals. The latter samples were dredged from our ten collecting stations established in the Sound. All these stations, located at a depth ranging from 10 to 30 feet at the mean low water stage, were subjected to strong tidal currents. The exact time of the collection of each sample was recorded and later correlated with the stage of the tide.

The collection of samples from all the stations extending along the shore for a distance of about 25 miles usually required from six to eight hours. Thus, it was sometimes possible to continue to collect samples during the entire period of the outgoing or incoming tide. The stations were not always attended in the same sequence to guarantee that the sampling was of a random nature.

The results of the examination of 1000 oysters offered rather conclusive evidence that the mollusks were feeding as actively on the ebb tide as they did during the flood (Table I). As a matter of fact, the per cent of oysters containing a large quantity of food was somewhat higher on the ebb than during the flood. Furthermore, only 6 per cent of the oysters collected during the ebbing tide possessed empty stomachs, while among the individuals dredged during the flood 10 per cent showed a complete absence of food. It is significant that among the oysters collected during the low water stage the per cent of the individuals containing a large quantity of food was higher, and that of the animals with empty stomachs lower than at many other stages of the tide. While this observation cannot be interpreted as definite proof that oysters feed most efficiently just prior and during the low water stage, it shows, nevertheless, that they do not cease, or noticeably decrease, their feeding activities during late ebb.

TABLE I

Relative quantities of food in stomachs of 1000 oysters collected at different tidal stages in Long Island Sound, June, July, and August 1945

Stage of tide	Oysters examined	Quantity of food			Per cent		
		Large	Small	Absent	Large	Small	Absent
Flood							
1st hour	41	31	6	4	75	15	10
2nd hour	60	51	2	7	85	3	12
3rd hour	85	58	9	18	68	11	21
4th hour	75	66	5	4	88	7	5
5th hour	85	72	8	5	85	9	6
High water	110	91	9	10	83	8	9
Total	456	369	39	48	81	9	10
Ebb							
1st hour	80	66	11	3	82	14	4
2nd hour	80	69	7	4	86	9	5
3rd hour	120	102	10	8	85	8	7
4th hour	90	76	10	4	85	11	4
5th hour	107	84	13	10	79	12	9
Low water	67	59	6	2	88	9	3
Total	544	456	57	31	84	10	6
Grand total	1000	825	96	79	82	10	8

Additional observations on the relative quantities of food in the stomachs of the oysters at the end of the flood and during the entire period of ebb were made in Long Island Sound on August 21, 1945. On that day a station was chosen in 20 feet of water on one of the beds planted with 4-year-old mollusks. The location of the station was designated by a special buoy. The samples were collected at hourly intervals beginning one hour prior to the high water stage (Table II). Altogether eight samples, each composed of 20 oysters, were collected and examined.

The salinity of the water ranged from 27.31 p.p.t. soon after high water to 25.45 p.p.t. at low water.

The results of the examination again showed that in the majority of the oysters the stomachs contained large quantities of food during all stages of the ebb (Table II). The observations also indicated that there was no decrease in the number of oysters with food-filled stomachs parallel with the falling of the tide. On the contrary, the largest number of oysters containing large quantities of food was found only one hour prior to low water.

It is significant that among the oysters examined during the last three hours of the ebb not a single individual was found with an empty stomach. While the presence of food in the oysters dredged during the early stages of the outgoing tide could be possibly explained by assuming that this food was ingested during the last hour of flood, such an explanation cannot be offered for the presence of food in the oysters examined from three to six hours after the high water stage. Our observations performed under laboratory conditions on oysters kept in water of 20.0° C. showed that these mollusks pass the particles of food through their entire digestive system from 1 hour 20 minutes to approximately 2 hours and 30 minutes. Therefore, it seems rather improbable that the food found in the oysters during the latter part of the ebb was that ingested during the late stage of the flood, four to six hours prior to examination.

TABLE II

Relative quantities of food in stomachs of 160 oysters collected at hourly intervals during last hour of flood and during ebb from a station established in 20 feet of water in Long Island Sound, August 21, 1945. Each sample composed of 20 oysters.

Stage of tide	Temperature °C.	Quantity of food		
		Large	Small	Absent
Flood				
5th hour	21.2	11	8	1
High water	20.9	17	3	0
Ebb				
1st hour	21.1	14	6	0
2nd hour	21.0	19	1	0
3rd hour	20.9	17	2	1
4th hour	21.1	19	1	0
5th hour	20.6	20	0	0
Low water	20.4	16	4	0
Total		133	25	2

In general, the observations made on 160 oysters on August 21, 1945, showed that the oysters of Long Island Sound fed very actively during the ebb, and that the relative quantities of food found in their stomachs during that period were at least equal to or even exceeding those recorded during the last hour of the preceding flood.

Although observations in Long Island Sound have demonstrated that there was no correlation between the stages of the tide and the quantities of food found in the stomachs of the oysters, it was desirable to supplement the data already available with additional observations on the oysters living under ecological conditions rather different than those of Long Island Sound proper. An area in Milford Harbor near the dock used for laboratory needs, was chosen for these observations. A large number of oysters living on the bottom near the dock made such an arrangement especially convenient.

Milford Harbor was selected because it was a typical example of a small partially inclosed body of water where extensive natural beds had existed. In recent times many of the beds were destroyed by overfishing, and the profile of the bottom was markedly changed by the dredging of a wide and deep channel. Nevertheless, the oysters quickly reestablished themselves in more shallow sections of the Harbor, and at present are quite common. A good setting of oysters regularly occurs in the Harbor, indicating that the conditions are favorable for the propagation of these mollusks. Usually changes in the temperature and salinity of the Harbor water during the tidal cycle are more pronounced than in Long Island Sound proper, where both these factors remain very steady (Loosanoff and Engle, 1940).

The observations consisted in examining the stomach content of the oysters at hourly intervals throughout a 24-hour period. The samples, each composed of six individuals, were suspended one day prior to the beginning of the examination in baskets made of 2-inch mesh poultry wire. All the baskets were kept at the same depth, namely, one foot below the mean low water line. They were separated from each other by a distance of approximately one foot and, therefore, the removal of any of the baskets did not disturb the oysters of the other containers. The experiment continued from 7:30 A.M. of July 27 until 8:00 A.M. of July 28, covering three low and two high water stages, and including periods of light and darkness (Table III). During this period the temperature of the water ranged from 22.0 to 25.0° C., the salinity fluctuated between 22.68 and 28.44 parts per thousand, and the pH from 7.7 to 8.7.

The data obtained indicated that during the two flood and two ebb periods covered, the majority of the oysters contained large quantities of food. Of the total number of 150 oysters examined 86 per cent belonged to that group. This figure closely approached that for the Long Island Sound oysters where 82 per cent were found to possess large quantities of food (Table I). Only 4 per cent of the Milford Harbor oysters were found with empty stomachs, the figure being too low to suggest that large groups of the oyster population ceased feeding for appreciatively long intervals during the period of observation.

More detailed studies of the data given in Table III do not offer sharply defined evidence which would lead to the conclusion that the oysters collected during the flood contained more food than those collected at ebb, or vice versa. Although it is true that all but one oyster collected during the second flood period, extending from 8:40 P.M. to 1:58 A.M., contained large quantities of food, virtually the same observations were made during the preceding period of ebb when 32 out of 36 oysters showed full stomachs. In each case only one oyster with an empty stomach was found. If the condition of the oysters during the first flood period (8:30 A.M. to 1:30 P.M.) is compared with that of the oysters examined during the second ebb period (3:00 A.M. to 8:00 A.M.), it will be found that in each case 28 individuals showed large, and 6 showed small quantities of food, while 2 oysters were with empty stomachs.

TABLE III

Temperature, salinity and pH of the water, and relative quantities of food in stomachs of oysters examined at hourly intervals during a 24-hour period in Milford Harbor on July 27 and 28, 1945. Each sample of oysters consisted of six individuals.

Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food		
					Large	Small	Absent
Low water	7:30 A.M.	24.2	22.68	7.7	6	0	0
Flood							
1st hour	8:30 A.M.	24.0	27.74	7.7	4	2	0
2nd hour	9:30 A.M.	23.2	26.59	8.0	3	2	1
3rd hour	10:30 A.M.	23.3	27.65	8.0	6	0	0
4th hour	11:30 A.M.	23.0	28.44	8.0	3	2	1
5th hour	12:30 P.M.	23.1	27.65	8.1	6	0	0
High water	1:30 P.M.	23.1	26.33	8.2	6	0	0
Ebb							
1st hour	2:30 P.M.	23.2	27.65	8.2	6	0	0
2nd hour	3:30 P.M.	23.0	28.51	8.3	6	0	0
3rd hour	4:30 P.M.	23.4	26.39	8.3	4	2	0
4th hour	5:30 P.M.	23.9	26.13	8.3	5	0	1
5th hour	6:30 P.M.	24.9	24.52	8.1	5	1	0
Low water	7:40 P.M.	24.7	24.13	8.4	6	0	0
Flood							
1st hour	8:45 P.M.	24.5	24.40	8.7	6	0	0
2nd hour	9:45 P.M.	23.9	25.73	8.5	6	0	0
3rd hour	10:45 P.M.	23.3	26.00	8.5	6	0	0
4th hour	11:40 P.M.	23.3	26.00	8.5	5	0	1
5th hour	12:45 A.M.	23.1	26.54	8.5	6	0	0
High water	1:58 A.M.	23.0	26.27	8.5	6	0	0
Ebb							
1st hour	3:00 A.M.	23.0	26.00	8.5	5	0	1
2nd hour	4:00 A.M.	23.1	26.27	8.5	6	0	0
3rd hour	5:00 A.M.	23.3	26.27	8.4	4	2	0
4th hour	6:00 A.M.	23.0	25.73	8.1	3	2	1
5th hour	7:00 A.M.	23.2	25.47	8.1	5	1	0
Low water	8:00 A.M.	23.4	25.47	8.0	5	1	0
Total					129	15	6
Per cent					86	10	4

Perhaps it should be emphasized that among the 18 animals collected at the three low water stages (Table III) all but one oyster had large quantities of food in their stomachs.

Studies of the same nature as those made on July 27 and 28 were performed again on August 9. However, in the latter case observations were made only during one flood and one ebb stage covering a period of about 12 hours. The results of the observations are incorporated in Table IV together with data on the water temperature, salinity and pH recorded at the collection of each sample. The results showed that more oysters with large quantities of food were found in the group collected during the ebb than among the animals examined during the flood. However, no significant difference was noted between the two groups in the number of oysters with empty stomachs.

TABLE IV

Relative quantities of food in oysters examined at hourly intervals during a 12-hour period in Milford Harbor on August 9, 1945. Each sample composed of 6 oysters. Temperature, salinity and pH are indicated for each stage of the tide.

Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food		
					Large	Small	Absent
Flood							
1st hour	7:55 A.M.	22.2	24.99	7.9	0	5	1
2nd hour	8:55 A.M.	21.9	25.72	7.9	1	5	0
3rd hour	9:55 A.M.	21.6	25.72	7.9	3	3	0
4th hour	10:55 A.M.	21.6	25.99	7.9	3	1	2
5th hour	11:55 A.M.	21.8	25.44	7.9	6	0	0
High water	12:55 P.M.	21.7	25.72	7.9	5	1	0
Total					18	15	3
Ebb							
1st hour	1:55 P.M.	22.0	25.72	8.0	4	2	0
2nd hour	2:55 P.M.	21.8	25.72	8.1	3	2	1
3rd hour	3:55 P.M.	22.0	25.72	8.1	6	0	0
4th hour	4:55 P.M.	23.9	25.44	8.1	6	0	0
5th hour	5:55 P.M.	24.4	23.84	8.0	4	2	0
Low water	7:05 P.M.	25.1	21.44	8.0	6	0	0
Total					29	6	1

In summarizing all our observations on the relative quantities of food present in the stomachs of the oysters during different tidal stages the conclusion may be formed that, as far as ingestion of food is concerned, the oysters of Long Island Sound and Milford Harbor do not show a definite preference either to ebb or to flood. During either stage the predominating majority of the oysters contained large quantities of food, whereas individuals with empty stomachs were found only occasionally. The data obtained fail to lend any support to the unqualified opinion that the American oyster, *O. virginica*, takes relatively little food on the ebb tide.

Rate of water pumping in relation to tide

To determine the rate of water pumping of oysters and, therefore, the efficiency of their feeding at different stages of the tide an apparatus was constructed which permitted the measurement of the actual quantities of water passing through an oyster (Fig. 1). This apparatus was installed on the edge of the dock situated along the western shore of Milford Harbor where a swift tidal current flowed unobstructedly. The water supply was obtained through the hose, *A*, being pumped by the pump, *B*, into the storage barrel, *C*. The intake end of the hose, protected by a screen of large mesh always remained in the same position, six inches above the bottom. A constant water level in the barrel, *C*, was maintained by allowing the excess water to escape through the overflow outlet, *D*, to which a length of hose was attached. The capacity of the pump was such that the water in the storage barrel

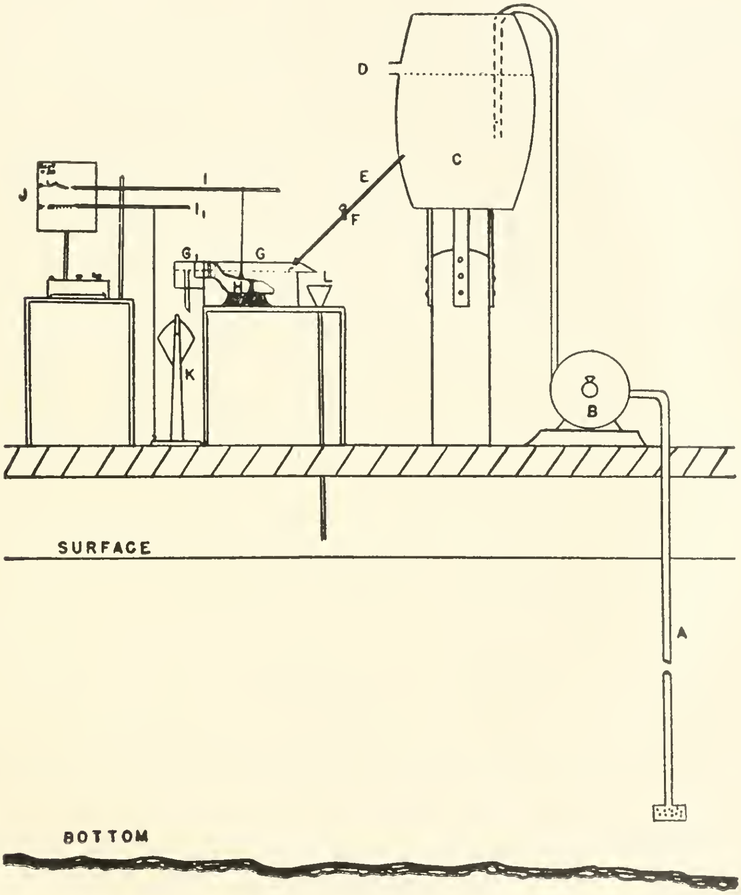

FIGURE 1. Diagram of the apparatus used in determining the quantities of water pumped by the experimental oysters. Description in text.

was renewed every 4 or 5 minutes. Thus, the experimental oysters were receiving water, which was changing parallel with the changes of the tide.

The water was fed to the experimental oysters through the tube, *E*, provided with a flow-adjusting cock, *F*. The constant level oyster chamber, *G*, contained the oyster, *H*, the excurrent side of which was covered with a rubber, cone-shaped apron which conducted the water pumped by the oyster into the smaller chamber, G_1. Moore (1908) and Nelson (1936) were the first to apply the rubber apron, while Galtsoff (1926) devised and began to use the chamber. A string glued to the upper shell of the oyster was attached to the counter-balanced lever, *I*, which recorded every movement of the shell on the kymograph, *J*.

The water pumped by the oyster into the chamber, G_1, overflowed through the glass tube standpipe into the tripping vessel, *K*, of known capacity. When the vessel was filled with the water pumped by the oyster it tripped over, emptying its contents, and at the same time striking a string attached to the lever, I_1, which made a mark on the kymograph, *J*. Thus, each tripping was recorded, and because the capacity of the vessel was known, the quantity of water pumped by the oyster during certain intervals could be ascertained easily. The excess water entering the chamber, *G*, but not utilized by the oyster flowed out through the outlet, *L*. The part of the experimental apparatus containing the oyster chambers and the kymograph was kept in a small shed to protect the oyster from the effects of the sun and from possible disturbances caused by the wind.

The oysters were placed in the apparatus usually from one to two hours before high or low water. This time was allotted to the oysters to open their shells and to begin pumping water at a normal rate. This introductory period was not included in the analysis of the pumping activities of the oysters.

At the end of the introductory period, which usually coincided with the high or low water stage, observations were carried on for 12 or 13 hours, covering one ebb and one flood stage. In this manner records of 27 oysters were obtained. Fortunately, in almost all cases the oysters remained open continuously throughout the period of exposure. The experiments were conducted at temperatures ranging from 19.1 to 25.8° C., a range considered very favorable for the pumping activities of oysters (Galtsoff, 1928).

In analyzing the kymograph records obtained in the course of these studies it was found convenient to divide each flood or ebb period into six hourly subperiods. However, since the ebb and flood periods of Milford Harbor are not usually of exactly 6-hour duration, the data for the last hour of each stage had to be arrived at by determining the quantity of water pumped by the oyster from the end of the fifth hour until the change of the tide and then calculated for a 60-minute period.

Analysis of the data showed that, in general, the rate of pumping of the oysters during the flood stages was somewhat slower than that during the ebbing periods (Table V). However, because of significant differences in the rate of pumping shown by individual oysters within the same hour of a tidal period, and after considering all the aspects of the data secured the opinion was formed that the oysters of Milford Harbor feed actively at all stages of the tide, and that the rate of feeding during the ebb is at least equal to and sometimes may be even more rapid than during the flood.

In Milford Harbor the strongest tidal currents occur in the middle of the period between the high and low water stages. This period, therefore, corresponds to the third and fourth hours of each stage. There is no evidence, nevertheless, that during this period the pumping of the oysters was more energetic than during the preceding or successive periods (Table V). It is of interest to note, however, that during the two last hours of the ebb the rate of pumping was somewhat accelerated.

The rapid rate at which many of the experimental oysters pumped water during ebb is well demonstrated in the photograph of the kymograph record showing the rate of pumping and shell movement of one of the experimental animals (Fig. 2). The period of observation lasted from 9:34 A.M. until 10:13 P.M., September 12, covering one complete flood and ebb period. Each vertical line of the lower record was made by the tripping vessel (Fig. 1), the capacity of which was 255 cc. Examination of the lower part of the record, which, incidentally, was made with the help of a dissecting microscope, a method always employed when the pumping was rapid and the marks on the kymograph were made close to each other, provided the following information: the minimum quantity of water pumped by the oyster during a single hourly period of the flood was 4080 cc., and the maximum, 20,655 cc. The average hourly rate of pumping for the entire flood period was 13,260 cc. For the ebb period these figures were 15,810, 23,715, and 20,244 cc. respectively.

TABLE V

Mean of quantities of water (in cubic centimeters) pumped by oysters during each hour of the flood and ebb periods. The data are based on the kymograph records of 27 oysters, Milford Harbor, Summer of 1945.

Stage of tide	Mean	Stage of tide	Mean
Flood		Ebb	
1st hour	15,952±1,214	1st hour	15,384± 787
2nd hour	14,411±1,578	2nd hour	15,962± 830
3rd hour	12,875±1,427	3rd hour	16,186±1,021
4th hour	12,470±1,389	4th hour	17,458±1,195
5th hour	13,438±1,130	5th hour	17,916±1,063
6th hour	14,131±1,040	6th hour	17,577± 941

Examination of other records of the same series revealed that during the ebb some of the oysters averaged from 25 to 27 thousand cc. per hour, while the maximum rate of pumping in some instances ranged from 31 to 34 thousand cc. per hour. Having in possession a large number of kymograph records of this nature one may well be inclined to disagree with the opinion that the American oyster is relatively inactive during the outgoing stage of the tide.

In connection with this discussion an interesting deduction can be made concerning the efficiency of the pumping mechanism of oysters. The average weight of the oyster meat removed from the shell the length of which is 4 inches is approximately 20 grams and its volume is usually not more than 20 cc. This organism, nevertheless, is capable of pumping 30 thousand cc. or more of sea water per hour. In other words, the volume of water passing through the oyster gills in one hour may be more than 1500 times greater than the volume of the oyster's body, a fact well attesting the efficacy of the feeding mechanism of this mollusk.

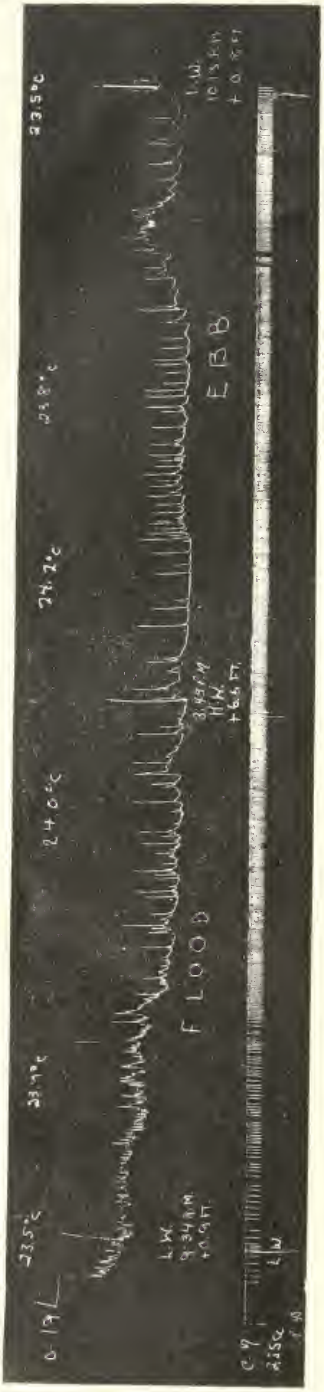

FIGURE 2. Photograph of kymograph record showing the shell movements (upper line) and rate of pumping (lower line) of oyster no. 19 obtained during the flood and ebb stages in Milford Harbor on September 12, 1945. Each vertical line of the lower record designates emptying of the tripping vessel (Fig. 1) of 255 cc. capacity. *L. W.* and *H. W.* indicate the low water and high water stages respectively.

Opening and closing of shells in relation to tide

Experiments for determining the presence or lack of correlation between the opening and closing of the oyster shells in relation to the tidal stages were also conducted in the Summer of 1945. The method used was the same as that successfully employed in the studies of the shell movements of hard shell clams, *Venus mercenaria,* and of the edible mussel, *Mytilus edulis* (Loosanoff, 1939, 1942). A description of this apparatus has already been published in full (Loosanoff, 1939) and, therefore, need not be repeated. It will be sufficient to say that this apparatus permitted registering on the Foxboro recorder of every shell movement of oysters kept under virtually natural conditions on the platform installed on the small oyster bed existing on the bottom of Milford Harbor. Even at low tides the experimental oysters were covered with at least 2 feet of water.

The area where the apparatus was installed was subjected to strong tidal currents which, during the spring tides, attained a velocity of 1.2 feet per second on the flood, and 1.5 feet per second on the ebb. During the neap tides, however, the velocity of the flood current was only 0.8 foot per second, and that of the ebb current, 1.3 feet per second. The mean range of tide in Milford Harbor is 6.6 feet, but during the spring tides high water occasionally reaches the 9-foot mark. In Milford Harbor, the same as in Long Island Sound, the strongest tidal currents occur in the middle of the period between high and low water. The slack usually coincides with the time of the high and low water stages.

Usually the records of two oysters were taken simultaneously—each record covering a 24-hour period. The temperature of the water was also continuously registered by Brown's recording thermometer which was installed near the shell movement recording apparatus, the bulb of the thermometer being only 5 inches away from the experimental oysters. The temperature range extended from 17.0 to 28.0° C., averaging approximately 22.0° C. Altogether 64 records of 18 different oysters were obtained in the course of these observations.

That the conditions in the Harbor were favorable for the existence of the mollusks was well shown by the growth of the young oysters which attached themselves to the concrete base of our apparatus used for studying the shell movements of the experimental animals (Fig. 3). The attachment, or, as it is more commonly called, the setting of the oysters took place early in August, soon after the apparatus was first placed in the water. The examination made late in September showed that the young oysters grew very well, regardless of the fact that the base was often removed from the water for short periods to change the experimental mollusks. This rapid growth of the young oysters, as well as their generally good condition, indicated that the experimental animals, the shell movements of which were recorded, were also subjected to favorable environment.

Analysis of 64 complete records showed that on an average the oysters remained open 22 hours and 39 minutes, or 94.3 per cent, and closed 1 hour and 21 minutes, or 5.7 per cent of a 24-hour period. Our figure for the duration of openness of the oyster shells is quite close to that of Nelson (1921) who, basing his conclusions on the records of 3 oysters, found that these animals remained open on an average of 20 hours per day, but is much higher than that offered by Galtsoff (1928) who found that the average period the oysters remain open is 17 hours and 7 minutes per day. However, the difference between Galtsoff's figures and ours may be explained on the basis that his observations were made on the oysters placed in the aquarium, while our animals were kept on the bottom of the Harbor, where conditions were different from those of the laboratory. Nevertheless, Galtsoff expresses the opinion, which coincides with ours, that oysters have a tendency to keep the shells open as long as possible.

Further statistical studies of the data collected showed that during the periods of flood the shells of the oysters remained open on an average of 93.4 per cent of the time, whereas during the ebb periods the shells were open 95.2 per cent. Obviously, the difference between the two figures is rather insignificant, indicating that the behavior of the oysters is quite similar during the opposite stages of the tide.

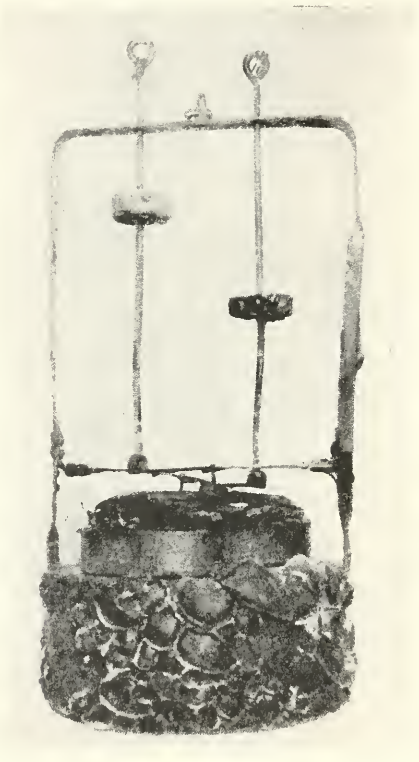

FIGURE 3. Photograph of the base of the instrument employed to record the shell movements of the oysters kept on the bottom of Milford Harbor. Note the healthy and vigorously growing young oysters attached to the sides of the base. Large experimental oysters imbedded in small concrete blocks can be seen on the top of the base.

Another trait of similar behavior of the oyster during the two different tidal stages was indicated by the fact that almost an equal number of records showing that the animals kept their shells open 100 per cent of the time was obtained for each stage. Thirty-three such records were made during the flood periods, and 32, during the ebb

Feeding in relation to time of day

While conducting these studies the opportunity presented itself to verify the statements that the feeding of oysters is considerably slowed down (Nelson, 1921, 1923) or entirely ceases (Orton, 1929) at night and in the early morning. The material for studying this problem was available from all three phases of our investigation, namely, observations on the stomach content of oysters, rate of pumping, and opening and closing of the shells.

Examination of the stomach content of the oysters made at hourly intervals during a 24-hour period in Milford Harbor on July 27 and 28, 1945, has shown that during the period of darkness, which extended from 8:33 P.M. until 5:33 A.M. (E.W.T.), the oysters contained as much food as during the hours of light (Table III). During the largest part of the period of darkness, until 5:00 A.M., almost all the oysters contained large quantities of food. The percentage of individuals with full stomachs during this period would compare favorably with that of the animals examined during the daytime. Numerous other examinations of the stomach content of the Milford Harbor oysters made between 1:00 and 6:00 A.M. during the Summer of 1945 also showed that the majority of the oysters had full stomachs. Obviously, these observations do not offer any support to the conclusion that oysters do not feed at night.

The results of the experiments in which the rate of pumping of the oysters was determined also contradict the above mentioned conclusion. Observations performed at night showed that the oysters remained active and fed very vigorously. The results of the experiment conducted during the night of July 27–28 were especially significant because in that case the period of late night coincided with the ebb (Fig. 4). Thus, the effects of two presumably unfavorable factors were combined. Nevertheless, as is shown in Figure 4, both experimental oysters remained open and continued to pump water at a very rapid rate during the entire period of darkness. When one of the animals closed at about 6:30 A.M. it already was one hour after sunrise. During the period of darkness the average rate of pumping of oyster no. 92 (upper record) was 17,934 cc. per hour, and the maximum, 19,272 cc. per hour. For oyster no. 98 (lower record) these figures were 24,622 and 30,600 cc. respectively.

Because this experiment was of a 24-hour duration data were also available on the rate of pumping of the same two oysters in daylight. The average rate of pumping of oyster no. 92 during that period was found to be 12,684 cc. per hour, and the maximum, 19,053 cc. per hour. For the second oysters the corresponding figures were 24,565 and 31,875 cc. Thus, in this case, the same as in the case of studies of the stomach content of the oysters, the conclusion may be offered that oysters feed actively during darkness. Furthermore, the average rate of pumping at night was not lower than during the daytime.

A series of supplementary experiments on the effect of light and darkness upon the rate of pumping also failed to show a significant difference in the behavior of oysters. These experiments were conducted in a dark room, where an apparatus of the type shown in Figure 1 was installed. Three or four oysters were tested simultaneously. During the period of light the oysters were illuminated with small floodlights. The intensity of light at the surface of the oysters, as determined by the Weston photronic exposure meter, was in excess of 1000 candles per square foot.

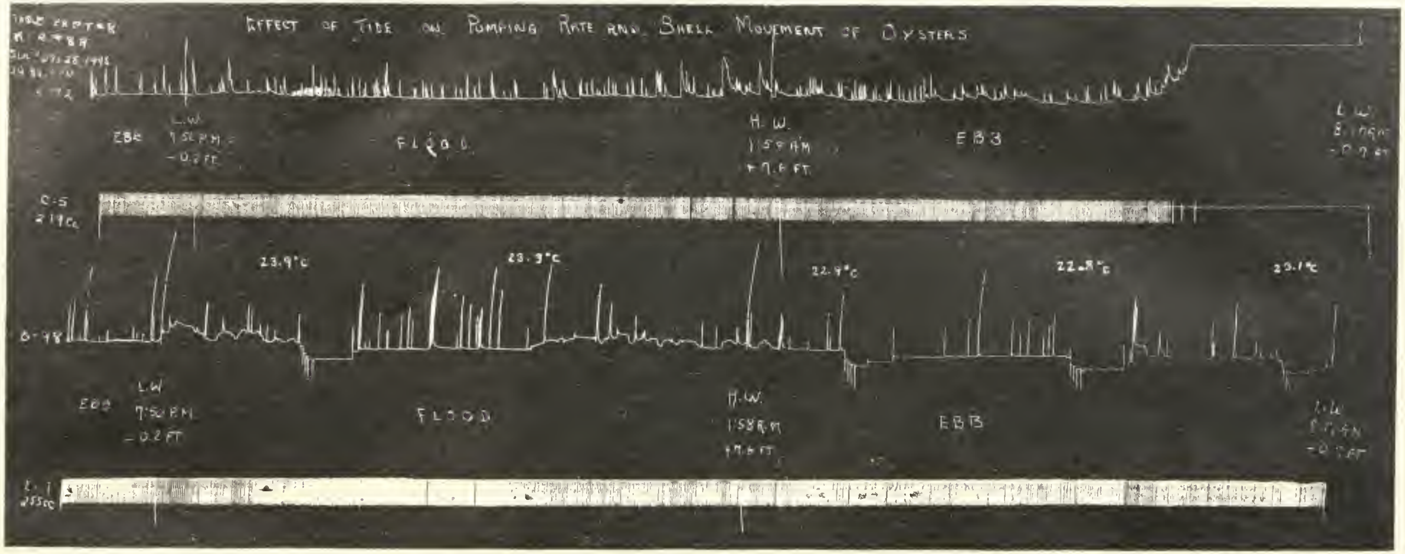

Figure 4. Photograph of the kymograph record showing the shell movements (1st and 3rd lines) and the rate of pumping (2nd and 4th lines) of two oysters during the period extending from 7:50 P.M., July 27 until 8:17 A.M. July 28, 1945. Each vertical mark of the second line designates emptying of the tripping vessel of 219 cc. capacity, while each mark of the fourth line shows the dumping of 255 cc. of water. The records show that both oysters fed very actively during the period of darkness, which extended from 8:33 P.M. until 5:33 A.M. *L. W.* and *H. W.* indicate the low water and high water stages respectively.

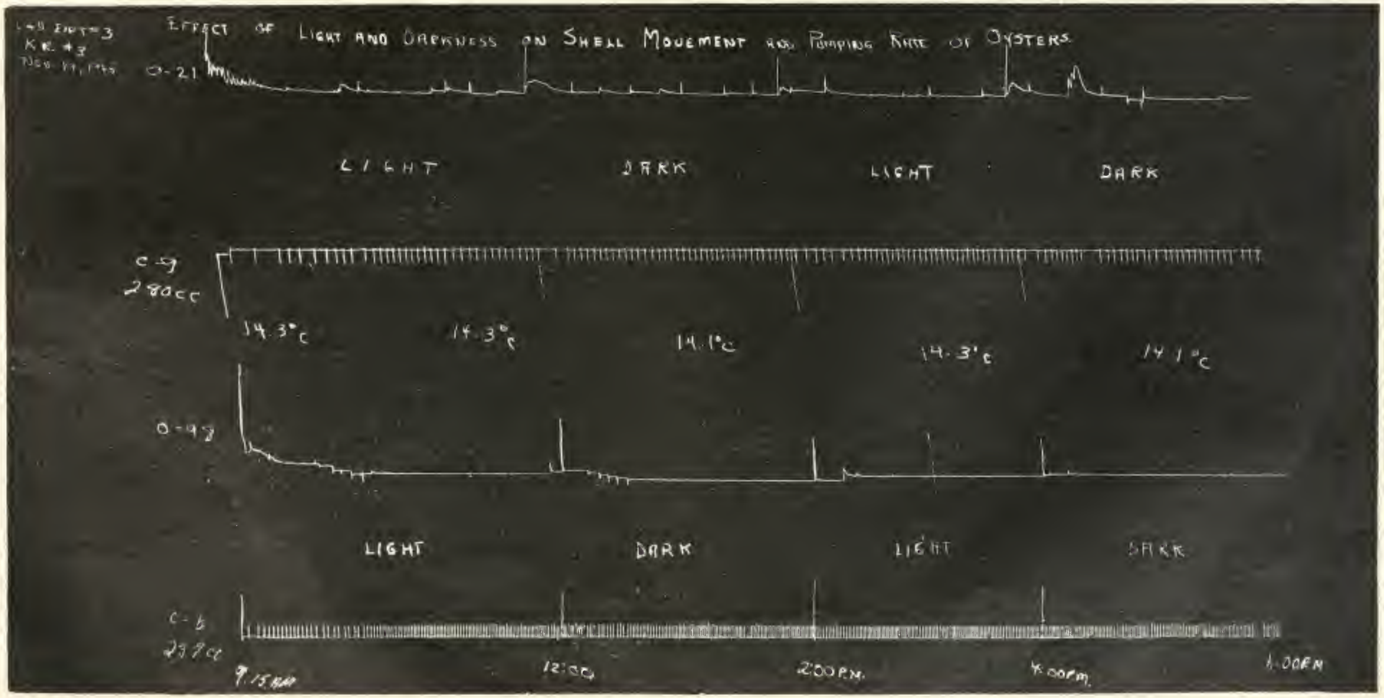

FIGURE 5. Photograph of kymograph record showing the shell movements (1st and 3rd lines) and the rate of pumping (2nd and 4th lines) of two oysters exposed to alternating periods of light and darkness. Each vertical mark of the second line designates the emptying of the tripping vessel of 280 cc. capacity, while each mark of the fourth line shows the dumping of 237 cc. of water.

The oysters were kept in the apparatus for approximately 9 hours (Fig. 5). During this time the mollusks were exposed to two periods of light and two of darkness. However, the initial period, of approximately 3 hours, was not included in the analysis, as this time was given to the oysters to become accustomed to the experimental condition. To equalize the data half of the experiments began with a period of light, and the other with that of darkness. Altogether 30 records of 10 different oysters were obtained. Analysis of the data showed that the mean hourly rate of pumping during the periods of light was 5350 ± 445 cc. For the periods of darkness this figure was 5036 ± 385. Thus, the rate of pumping during periods of light closely resembles that during periods of darkness.

Finally, the data on the same subject were taken from our 64 daily records of the shell movements of oysters. Analysis of these data showed that on an average the shells of the oysters remained open 94.4 per cent of the total time during daylight, and 93.8 per cent during the periods of darkness. The difference of less than one per cent is not considered as significant in this case, and, therefore, it may be concluded that in our experiments no correlation was found between the periods of closure of the shells and darkness. These results are in agreement with those of Galtsoff (1928) who in his article disagrees with Nelson's (1921, 1923) conclusion that the period of darkness, between 11:00 P.M. and 4:30 A.M., should be considered as a time of rest for oysters.

Discussion

The advocates of the opinion that oysters considerably reduce their feeding activities during the periods of ebb failed to suggest in their publications any factors which could be considered as responsible for the change in the behavior of the mollusks. It could be easily understood that in some areas, where the periods preceding and coinciding with the low water stage are accompanied by distinctly unfavorable changes in the environment, the oysters would temporarily slow down or even cease feeding. For example, a sharp decrease in salinity could compel the oysters to be relatively inactive. However, according to Nelson (1921) the reduction in salinity was not the cause. This conclusion is based upon his statement that in Huey's Creek, where the experiments were conducted, the periods of complete cessation or of the commencement of feeding, although showing a definite correlation with the stage of the tide, occurred independently of the changes in the density of the water, because such changes were usually of small magnitude.

Changes in the turbidity and temperature of the water were also considered as unimportant by Nelson (1923), who concluded that "The rate of filtration of water during any given period of time, as deducted from the rapidity and extent of ejections of accumulated sediment from the mantle cavity, may vary widely independently of the temperature and the turbidity of the water." In the same article Nelson also stated that "No correlation could be shown between the food content of the water and the periods of inactivity of the oyster." All these conclusions were based upon the experimental data first reported by Nelson in 1921. Thus, according to that author, neither changes in salinity or temperature, nor changes in the turbidity or quantity of food present in the water affected the rate of feeding of oysters. Yet, because of some undetermined factors these mollusks fed much less actively during the ebb stages.

In discussing Nelson's (1921) work it is necessary to mention that his experiments were devised to study the shell movements of the oysters, but not the rate at which these mollusks were filtering water through the gills, i.e., feeding. Only the shell movements of the oysters were recorded on the kymograph, while no data were obtained on the quantities of water pumped by the oysters during the different tidal stages. Obviously, no definite conclusions could be formed concerning the latter subject because of the almost complete lack of experimental evidence regarding this matter.

Nelson's method of interpreting the data should also be mentioned. In analyzing his material on shell movement of the oysters Nelson (1921) takes into consideration only the numbers of openings and closures of the shells during the different tidal periods. This method has already been criticized by Galtsoff (1928) who stated that "The examination of the number of closures and openings occurring during a given period of time does not convey a true idea of the activity or inactivity of the oyster. A better understanding can be gained by counting the number of hours the oyster was closed or open during a given period of a day." Obviously, Galtsoff's suggestion is well founded.

Our observations and experiments supplied the evidence that the oysters of Long Island Sound and Milford Harbor fed actively during the flood and ebb periods, and that during the ebb their feeding was often more energetic than during the flood. Observations of this nature could probably be made in many other bodies of water where changes in the tides are not accompanied by pronounced ecological changes. This conclusion appears to be logical because it is quite improbable that, if other conditions of the environment remain favorable, a change in the direction of the tidal current alone would affect the oysters. It is, to a certain extent, supported by our experiments in which pairs of oysters, employed in our studies of the shell movements, were always placed so that the hinges of their shells pointed in opposite directions. Thus, while the gills of one oyster faced the flood, the gills of the other animal were turned away from the direction of the current. Yet, no difference suggesting that an oyster in a certain position was more active on the flood or ebb was generally noted.

No correlation between the rate of water pumping of the oysters and the time of day was demonstrated by our experiments. Neither was it found that the duration of the opening and closure of the shells was affected by periods of light or darkness. In nature many oyster beds are located at a considerable depth. Very often the water flowing over the beds contains large quantities of suspended matter which stop the penetration of a large quantity of light before it reaches the bottom on which the oysters live. These oysters, therefore, normally exist in near-darkness even during very strong daylight. It is very doubtful that if other conditions remain favorable, the slight change in the intensity of illumination caused by the approach of night could have such a pronounced effect on the oysters that they would either begin to feed at a much slower rate or stop feeding entirely.

If the rate of feeding of oysters were markedly decreased during the nights and during the ebb periods, the existence of these mollusks would be under a rather unfavorable condition. Because the periods of darkness are often followed by ebb, there would be times when the feeding activities of the oysters would be continuously depressed for a period of approximately 18 hours. This condition would occur in September and October when the nights become long. However, it is a well known fact that during these two months the oysters of our waters undergo very rapid improvement in condition storing large quantities of glycogen in their bodies. Naturally, such an improvement could not be possible if, during this time of the year, the oysters had to exist under the conditions compelling them to be relatively inactive during approximately 12 hours of darkness and also during the 6 hours of ebb, a total of 18 hours per day.

As may be seen from this discussion, our conclusions regarding the activities of oysters during ebb and during periods of darkness do not agree with those of Nelson and Orton (ref. cit.). However, as Dr. Nelson suggests in recent personal communication with the senior author, the cause of the divergence may be attributed to the marked reduction of the pH during ebb and at night in the waters where Nelson's experiments were conducted. During the outgoing tide those areas received large quantities of swamp water which noticeably lowered the pH. Also, according to Nelson "These waters are but slightly buffered; hence at night with the respiration of algae and of animals and decomposition the water may become acid by morning." Such changes are indicated in one of Nelson's reports (1924). The conditions, however, are different in other basins, such as in many sections of Chesapeake Bay (Loosanoff, 1932) and Long Island Sound (Loosanoff and Engle, 1940) where the pH does not closely approach the neutral point.

In general, our experiments have shown that under favorable conditions neither tidal changes nor changes in the time of day affect the rate of feeding of oysters of Milford Harbor and Long Island Sound. Although the differences in the behavior between the individual oysters are of considerable magnitude, these mollusks, nevertheless, appeared to be feeding all or most of the time their shells remained open which, with a temperature range from 17.0 to 28.0° C., was approximately 94 per cent of the total time.

In presenting the final conclusions it should be once more emphasized that we do not interpret our results as applicable to all oyster growing areas of this coast. While our observations hold true for the areas where the experiments were conducted, and also, probably, for the waters where the ecological conditions resemble ours, it is realized that in other basins, where during ebb the oysters are exposed to unfavorable environment, different conditions may prevail. Nevertheless, the material presented in this article clearly indicates that the conclusions of Nelson (ref. cit.), which, no doubt, are representative for Huey's Creek, should not have been generalized and presented as applicable to the American oyster as a species (Orton, 1929).

Summary

1. Examination of approximately 1400 oysters collected during the different tidal stages in Long Island Sound and Milford Harbor failed to show any definite period when the stomachs of these mollusks displayed absence of food.

2. During all hours of the flood and ebb, including the low water period, the predominating majority of the oysters contained large quantities of food, whereas individuals with empty stomachs were found only occasionally.

3. The relative quantities of food found in the oyster stomachs during the ebb period were at least equal to or sometimes even exceeded those recorded during the flood.

4. Analysis of the kymograph records of the rate of water pumping by the oysters showed that they fed very actively at all stages of the tide, and that the rate of feeding during ebb was at least equal to or sometimes even more rapid than during the flood stage.

5. During the ebb some of the oysters pumped on an average of 25,000 to 27,000 cc. of water per hour, while the maximum rate of pumping in some instances ranged from 31,000 to 34,000 cc. per hour.

6. The efficiency of the feeding mechanism of an oyster may be well attested by the fact that the volume of water passed during one hour through the oyster gills may be more than 1500 times greater than the volume of the oyster's body.

7. Within the temperature range of 17.0 to 28.0° C. the oysters remained open on an average of 22 hours and 39 minutes, or 94.3 per cent, and were closed 1 hour and 21 minutes, or 5.7 per cent of a 24-hour period.

8. During the periods of flood the shells of the oysters remained open on an average of 93.4 per cent of the time, whereas during the ebb periods the shells were open 95.2 per cent.

9. During the periods of darkness the percentage of oysters with full stomachs was comparable to that of the individuals examined during the day time.

10. During darkness the oysters were found feeding very actively. The average rate of pumping at night was not lower than during the daytime.

11. The shells of the oysters remained open 94.4 per cent of the total time during daylight, and 93.8 per cent during the period of darkness. No correlation was found between the periods of closure of the shells and darkness.

12. Under favorable conditions neither tidal changes nor changes in the time of day affect the rate of feeding of oysters of Milford Harbor. These mollusks were found to be feeding all or most of the time when their shells remained open.

13. The results of this investigation do not lend any support to the generally accepted theory that the American oyster does not feed late at night and in the early morning, and is relatively inactive on the ebb tide.

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AUTOSOMAL ELIMINATION AND PREFERENTIAL SEGREGATION IN THE HARLEQUIN LOBE OF CERTAIN DISCOCEPHALINI (HEMIPTERA)

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INTRODUCTION

It has been known for some time that the various lobes in the testis of many species of pentatomid Hemiptera show constant differences in the size of their cells. Bowen (1922a and b) who investigated this condition most recently, concluded that it is attributable mainly to differences in the volume of cytoplasmic elements. That is a finding which I can only confirm; if differences in the volume of chromatin exist they must be very small. However in some species there is a testicular lobe whose cells differ from those of other lobes not only in size but which has also evolved an entirely novel process of maturation. The main features of this maturation are in several instances almost fantastic in character, and the evolution and constant occurrence of such "harlequin" lobes is a matter of some interest.

It should be emphasized that we are dealing here not with accidental or sporadic occurrences. In the species concerned the harlequin lobe is found in each testis of every male. Moreover for any given species it is always a certain and very definite lobe that is thus characterized (in the Discocephalini here treated it is the fifth) and hence it is clear that its development involves conditions that are fundamentally and firmly established in the species as it is now constituted.

Harlequin lobes have so far been encountered in three species of Loxa, a species of Mayrinia (Schrader, 1945a and b), and in a species of Brachystethus (Schrader, 1946). In the last named the departure from a normal meiosis lies primarly in the autosomes which are shunted out of the spindle in both divisions; in Loxa and Mayrinia the aberrancy takes the form of amitosis and fusion in the spermatocytes, resulting in a highly variable heteroploidy. The meiotic anomalies of Brachystethus and Loxa thus appear to be in no way related and yet it seems only natural to assume as a working hypothesis that the evolution of harlequin lobes involves similar basic conditions in all the species involved.

It is likely that further investigations will discover that harlequin lobes are present in a great many species. To the five species mentioned above and the three taken up in the present paper may be added at least three further species which I have not as yet fully analyzed—a total of eleven. Taxonomically speaking, these species cover a wide range. Loxa and Mayrinia represent typical genera of the tribe of Pentatomini; Brachystethus is so closely related to the Edessini as to furnish almost a "bridging" genus between that tribe and the Pentatomini; and the Discocephalini constitute a tribe so distinct from the other pentatomid tribes that it has sometimes been elevated to the rank of a subfamily (Lethierry and Severin, 1896).

Conditions in the females of all these species are still unknown, except as they were used in all instances to check the identification of the sex chromosomes in the males. Cytologists need hardly be told that this gap in our knowledge is due mainly to the technical difficulties that render a study of meiosis in the egg so onerous a task.

I should like to point out again as I have done in my study of Brachystethus, that the investigation of the harlequin lobe is made under the almost ideal conditions of a natural experiment. The adjoining lobes of the same testis are perfectly normal and serve continually as a control; frequently the normal and aberrant cells can be studied in one and the same field of the microscope.

MATERIAL AND METHODS

The Discocephalini investigated are: *Mecistorhinus melanoleucus* Westwood (one male from Panama); *Mecistorhinus tripterus* Fabricius (four males and two females from Costa Rica); *Mecistorhinus sepulcralis* Fabricius (one testis each from eight different males and the ovaries from one female, all from Piracicaba, Brazil); *Neodine macraspis* Perty (five males from Costa Rica); and *Platycarenus notulatus* Stål (three males and one female from Costa Rica). The last named species has no harlequin lobe and is only briefly mentioned in the following pages.

My thanks are due to the eminent hemipterist, Mr. H. G. Barber, who identified all the species of Mecistorhinus. To Professor S. de Toledo Piza of the University of São Paulo, Brazil, I am deeply indebted for the material of *Mecistorhinus sepulcralis.*

Fixation was made in either Bauer's convenient modification of Allen's Bouin or in Sanfelice. As in all my recent studies of mitosis, I have employed three staining methods. The Feulgen technique is indispensable as a test for chromatin; gentian violet (in Smith's modification of Newton's method) is often very useful for a study of the detailed structure of the chromosomes but even when combined with erythrocin is not an efficient stain for the spindle apparatus in pentatomids; whereas Heidenhain's hematoxylin remains beyond all comparison the best means for bringing out asters, centrioles and spindle fibers. As noted above, female material was studied only to check identification of the sex chromosomes in the male.

In all the Discocephalini where a harlequin lobe occurs, it is the fifth of seven lobes in each testis. In every case it is two or three times as voluminous as any other lobe although its spermatocyte cells after the leptotene stage are smaller than those of the rest of the testis. The fourth and sixth lobes which flank it on either side carry exceptionally large but otherwise normal cells, whereas the remainder conform to more orthodox proportions.

In the following pages the different species are taken up separately, the detailed analysis of *Mecistorhinus melanoleucus* being followed by briefer comparative accounts of the other forms. As far as possible, the interpretative treatment is relegated to the discussion that terminates the paper.

MECISTORHINUS MELANOLEUCUS

Normal lobes

Except for certain features which are pertinent to an analysis of the peculiarities of the harlequin lobe, no detailed account of the spermatogenesis in the six normal lobes need be given. It conforms closely in its general course to that which has often been described in other pentatomids.

The diploid set of fourteen chromosomes is marked by one exceptionally large pair of autosomes. This pair stands out almost as conspicuously as does the X chromosome of Protenor. Here however, the sex chromosomes are relatively small, the Y being the smallest member of the complement and the X little if any larger than the smallest of the autosomes (Fig. 2).

One of the exceptional features lies in the heteropycnosis that marks not only the sex chromosomes but also certain of the autosomes. Already in the early generations of the spermatogonia there are from three to five heteropycnotic bodies that stand out prominently in the resting phase (Fig. 1). There is no prochromosome stage intervening between the last spermatogonia and the leptotene stage of meiosis. In the leptotene and synaptic stages the heteropycnotic bodies are usually aggregated in a single mass on the nuclear periphery and it is at this locus also that the fine leptotene threads come together in a bouquet formation (Fig. 3). But in some cells there are two heteropycnotic bodies during these stages, the second and smaller one usually lying at some distance from the first and not necessarily at the periphery. The advent of the pachytene and diplotene stages sees little change (Fig. 4) in these conditions of heteropycnosis and it is only when they in turn give way to the confused stage that the single heteropycnotic aggregate is dissociated again. In this peculiar phase when staining conditions and despiralization temporarily convert most of the chromosomes into pale and flocculent threads there may again be three, four, or five heteropycnotic bodies (Fig. 5). This variation in number would seem to indicate that the mutual and nonspecific attraction that brings heteropycnotic chromosomal bodies together at certain stages is not very strong and it is likely that accidents of position determine these numbers to some extent.

In early diakinesis, as the chromosome threads again become definite in outline, the topographic relationships are once more open to analysis. Now the great majority of cells show only two heteropycnotic bodies, one of which becomes less and less conspicuous as the threads shorten and condense[1] while the larger one is seen to be intimately associated with the big bivalent (Fig. 6). Somewhat later, when the paired chromosomes have assumed the typical cross and ring formations of late diakinesis this large heteropycnotic body has disappeared, but there are then two smaller bodies, one associated with each of the two spreading arms of the large bivalent (Fig. 7). There is no doubt about the identity of these bodies. The larger is the X and the smaller the Y, the two together constituting the larger heteropycnotic body or chromosome nucleolus of earlier diakinesis. The dissociation of this single nucleolus into its two components is perhaps due not only to the strains that attend the separation of the arms of the large bivalent but may be a part of the regular cycle that in other species also sees the reappearance of the separate sex chromosomes at this stage. What is more remarkable is their persistent union with the arms of the autosomal bivalent, a union which is not broken until shortly before metaphase.

The rather even peripheral distribution of the diakinetic bivalents disappears just before the disintegration of the nuclear membrane. They then lie helter skelter in the nucleus and may even come into contact with each other. It is at this time when the chromosomes are in the final stages of condensation that the two sex chromosomes sever their connections with the large tetrad, though their former association is frequently indicated by their close proximity to it (Fig. 8). It is this stage also that is marked by an elongation of the nucleus as a whole in the polar axis, a change that plainly involves interaction with the two centers located at the periphery of the cell.

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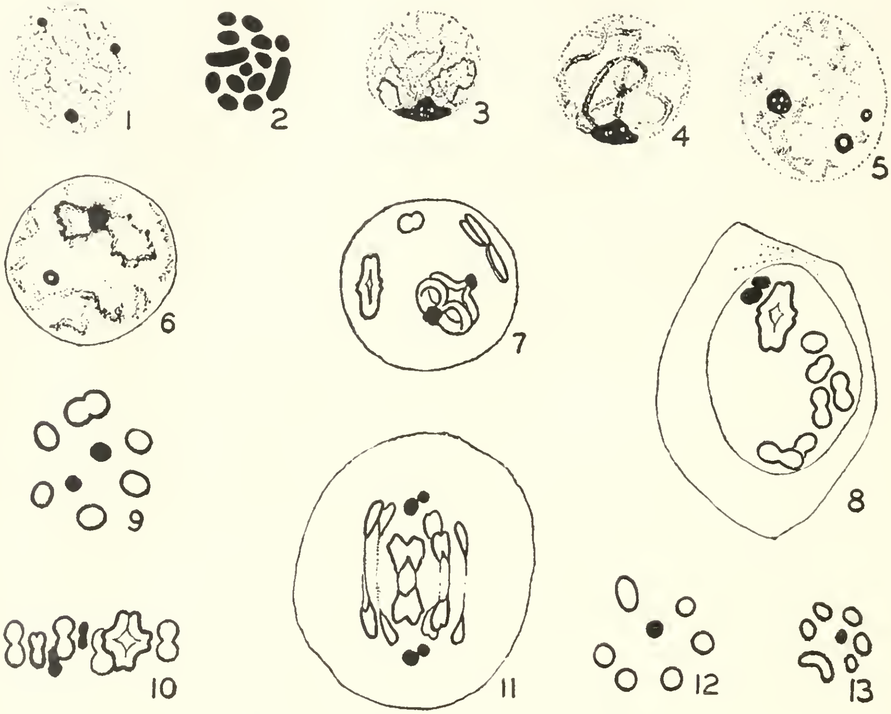
[1] The present state of our knowledge concerning the changes in the chromosome during a complete mitotic cycle is still unsatisfactory. Almost certainly both coiling and nucleination are involved, but the relative importance of these two factors remains undetermined. For that reason the terms "condensation" and "diffuseness" are here used in a purely descriptive sense.



Mecistorhinus melanoleucus—Normal Lobe

Figure 1. Early prophase in spermatogonial cell; three chromatin nucleoli (Feulgen).
Figure 2. Spermatogonial metaphase; Y is smallest of the 14 chromosomes (Feulgen).
Figure 3. Leptotene stage (Feulgen).
Figure 4. Diplotene stage (Feulgen).
Figure 5. Confused stage (Feulgen).
Figure 6. Early diakinesis; XY nucleolus attached to large tetrad (Feulgen).
Figure 7. Late diakinesis; X and Y attached to separate arms of large tetrad (Hematoxylin).
Figure 8. Prometaphase; X and Y still close to large tetrad (Hematoxylin).
Figure 9. Metaphase I; polar view (Hematoxylin).
Figure 10. Metaphase I; side view (Feulgen).
Figure 11. Anaphase I; large tetrad lagging (Gentian violet).
Figure 12. Metaphase II; polar view (Hematoxylin).
Figure 13. Telophase II; 6 autosomes + Y (Feulgen).

About the meiotic divisions themselves, little need be said. Metaphase I is quite typical in its conformation, with the now separated X and Y usually in the center of a ring of six tetrads (Fig. 9). It is however worthy of note that the large bivalent is somewhat slower than the rest of the autosomes in its condensation and side views of the first equatorial plate still show it as a cross tetrad (Fig. 10). At anaphase the X and Y divide equationally and arrive at the poles before the rest of the chromosomes, whereas the large bivalent (often showing the tertiary split) lags in its division and is distinctly slower than the other autosomes in its anaphasic progress (Fig. 11).

The second division also witnesses some lagging on part of the large autosome, but this is not as striking as in the first division. The touch and go pairing of the X and Y occurs as usual, and in metaphase they line up in the spindle axis so that in polar views one is superimposed on the other (Fig. 12). They then separate to oposite poles and the spermatids receive the typical pentatomid complements of 6A + X and 6A + Y respectively (Fig. 13). The departures from the orthodox process of meiosis thus do not affect the results, which conform to the regular pentatomid scheme.

Harlequin lobe

Spermatogonia and meiotic prophases

The spermatogonial stages in the harlequin lobe differ in no discernible way from those of the normal lobes. Here too there are, in Feulgen preparations, from three to five heteropycnotic bodies in the resting phase and the succeeding stages closely parallel the normal course of events. As in the normal lobes there is no prochromosome stage. The meiotic leptotene duplicates that of the other lobes in the number and disposition of heteropycnotic bodies, but the chromosome threads do not seem to be as finely drawn out and delicate as they normally are. This difference however is too slight to furnish a secure basis for contrast (Fig. 14). Succeeding this stage the developments follow a path that diverges widely from the usual one.

There is neither a synapsis nor a pachytene stage. During the period in which these developments occur in the normal lobes, the chromosome threads of the harlequin lobe merely abandon their bouquet orientation and undergo a progressive condensation. As a result the nucleus then shows twelve somewhat loosely coiled autosomes and the two more condensed sex chromosomes, clear evidence that any sort of pairing that may have occurred unobserved prior to this time has now been abrogated (Fig. 15). The picture presented is a surprisingly close approximation of the prochromosome stage as it occurs normally in some Hemiptera, and in this respect has some resemblance to the conditions in the harlequin lobe of Loxa (Schrader, 1945b) where such a post-leptotene condensation is also encountered. But in Loxa there is a true prochromosome stage as well which occurs quite normally prior to the evolution of the leptotene threads. Both there and in Mecistorhinus no confusion is possible for not only does the true prochromosome stage occur much higher in the testis, but its nuclei are considerably smaller than are the ones here in question.

This post-leptotene condensation culminates in shortened, fuzzy chromosomes that show an equational split (Fig. 16), undoubtedly a condition corresponding to

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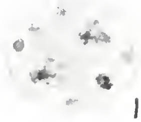
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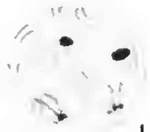
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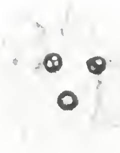
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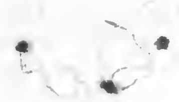
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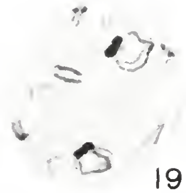
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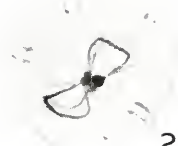
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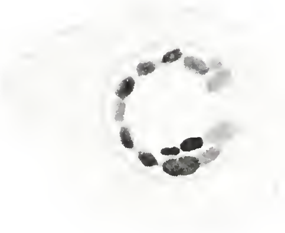
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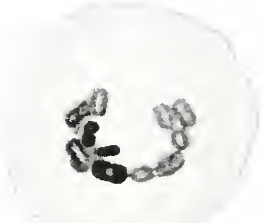
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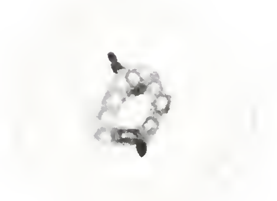
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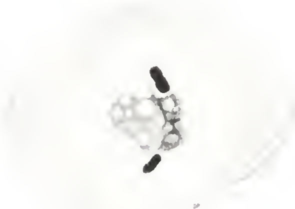
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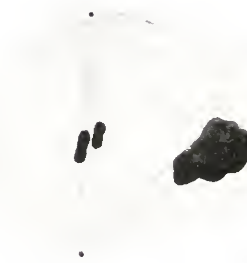
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Mecistorhinus melanoleucus—Harlequin Lobe

FIGURE 14. Leptotene stage (Gentian violet).

FIGURE 15. Post leptotene condensation; X and Y heteropycnotic (Feulgen).

FIGURE 16. Diplotene stage in univalents (Gentian violet).

FIGURE 17. Late confused stage (Feulgen).

FIGURE 18. Early diakinesis; sex chromosomes attached to separate large autosome (Gentian violet).

FIGURE 19. Mid-diakinesis; 12 univalents autosomes, with each large autosome combined with one sex chromosome (Gentian violet).

the diplotene stage of the normal lobes. Throughout this period two pairs of chromosomes are readily recognizable; they are the two large autosomes and the two heteropycnotic sex chromosomes. Each large autosome has its ends united so as to form a split ring, a configuration that very probably arises from the mutual attraction of its heteropycnotic terminal regions. The two heteropycnotic sex chromosomes show no such attraction at this time and usually lie well separated, evidence that heteropycnotic attraction is confined to certain conditions of the heterochromatin.

The confused stage which now intervenes, temporarily halts a further close analysis of progressive chromosome changes. The autosomes once more become diffuse and uncoiled and at the height of the stage stain very lightly. Usually three heteropycnotic bodies are present at this time, but there may be as many as five (Fig. 17). In the latter case the bodies are smaller, generally speaking, which would indicate that the variations in number are due to some vagaries in mutual attraction and aggregation. The two sex chromosomes and the ends of the two autosomes would account for six such bodies which suggests that some aggregation is nearly always present.

With the termination of the confused stage and the beginning of diakinesis, the individual chromosomes once more appear as such. There are then three heteropycnotic bodies and two of these are seen to be associated with the two large autosomes (Fig. 18). The third shows no such definite association and gradually disappears. In mid-diakinesis a more exact analysis of these conditions becomes possible. At this time there is a total of either eleven or twelve chromosomal bodies in every nucleus. When there are twelve, the two autosomes are quite independent of each other and may lie far apart. Each of them has a large chromatin nucleolus or heteropycnotic body attached to it at the place where the ends are still joined in ring formation (Fig. 19). When on the other hand there are only eleven bodies, these two chromatin nucleoli have come together, and through them the two large, ring formed autosomes have joined in a figure eight (Fig. 20). The two chromatin nucleoli represent the X and Y chromosomes and again, the most natural explanation of such configurations would seem to lie in the forces of heteropycnotic attraction; the heteropycnotic ends of the large autosomes are drawn together to form rings, and the heteropycnotic sex chromosomes later become attached to these regions and to each other for the same reason. It is rather strange that no case has been encountered in which both sex chromosomes have become joined to only one of the large autosomes, since nonspecific heteropycnotic attraction might be expected to give rise occasionally to such configurations. However, nuclei of this stage in which the chromosomes are open to a clear analysis are not common and

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FIGURE 20. Mid-diakinesis; both sex chromosomes and both large autosomes in one combination (Gentian violet).

FIGURE 21. Equatorial ring side view (Hematoxylin).

FIGURE 22. Equatorial ring slightly later; polar view (Gentian violet).

FIGURE 23. Autosomes in precocious return to diffuse condition; X and Y still heteropycnotic (Gentian violet).

FIGURE 24. Formation of autosomal aggregate; X and Y heteropycnotic (Gentian violet).

FIGURE 25. Dissociation of X and Y from autosomal aggregate (Gentian violet).

FIGURE 26. Metaphase I; X and Y on middle spindle and autosomal aggregate displaced (Hematoxylin).

the fourteen examples which have been studied hardly constitute a sufficiently large number to justify the conclusion that they do not occur.

Prometaphase

Shortly before the breakdown of the nuclear membrane a significant reorientation of the chromosomes takes place. This is at about the time that the nucleus elongates toward the peripherally located centers. The chromosomes, still not fully condensed, then are shifted to the middle region between the centers and since they remain in close proximity to the nuclear wall and have lost the property of mutual repulsion, they tend to form a more or less circular row or chain in the equator. Some of the components of such chains may be in actual contact with each other, while others may be connected by Feulgen positive bridges or show no attachment at all (Figs. 21, 22, and 72). It is likely that such bridges are similar in nature to those seen later at metaphase (see for instance Ris, 1942), but whether they represent viscous connections that persist after a former contact or are indicative of a "reaching out" of chromosomes toward each other, it is impossible to decide.

When the nuclear membrane finally disappears, this picture undergoes marked and sudden changes. The chain of chromosomes, now free of the influence of the membrane, seems to collapse inwardly, frequently forming a closed ring at first and then an irregular aggregate in the middle of the nuclear space. In the many cells seen at this and the following stages no instance of more than a single aggregate has ever been observed, a point of difference with the case of Brachystethus (Schrader, 1946). Concurrently with these changes of orientation there occur alterations in the chromosomal structure. These are marked especially by a partial return to the diffuse condition in the autosomes, with an accentuation of the equational split. The two large autosomes do not seem to become quite as diffuse as the rest, but this difference is not a striking one at best. This return to a more diffuse state causes the autosomal aggregate to appear as a spongy and vacuolated mass in both gentian violet and Feulgen preparations and this condition is maintained for the major part of the first division (Fig. 23). Hematoxylin slides allow no such structural diagnosis for there the aggregate is nearly always homogeneously and intensely stained.

The behavior of the sex chromosomes is remarkable during the prometaphase and the establishment of the metaphase itself. At the time of the equatorial ring formation, just prior to the disintegration of the nuclear membrane, they are still very close or even in contact with the large autosomes. Almost always they lie on the inner side of the ring and not in seriation with the rest of the chromosomes (Fig. 22). They seem to be almost fully condensed at this time and are recognizable in most cells. When the autosomal chain collapses to form the irregular aggregate, this distinction becomes even more marked, for in contrast to the autosomes they then maintain their condensed state and in addition tend to protrude from the spongy mass of autosomes (Fig. 24). This protrusion seems to be an indication of interaction with the two centers, for the sex chromosomes not only make their appearance on the side toward one of the poles but begin to place their long axis in alignment with the polar axis (Fig. 25) which is a placement assumed also at the ensuing metaphase.

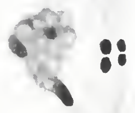
The first division

The clumping of the partially diffused autosomes brings about a rather anomalous situation and the establishment of the metaphase can be followed only through the behavior of the sex chromosomes. These appear to be quite normal in their further maneuvers. They finally become completely detached from the spongy aggregate of autosomes and take up an equatorial position side by side. During this movement several other developments occur simultaneously. Chromosomal fibers appear connecting the sex chromosomes as well as the aggregate with the poles, and at the same time the whole mass of autosomes is shunted out of the middle region toward the side of the cell. This shift must be rather sudden, for intermediate stages are very rare. In extreme instances the displacement may bring the autosomal aggregate very close to the side of the cell, though never touching it, and in every case it comes to lie farther from the polar axis than from the cell wall. While in this position, two points are to be noted: the autosomal aggregate remains connected with the poles through definite chromosomal fibers, and even in its displacement it maintains an equal distance from both poles (Figs. 26, 73, and 74). The whole reaction is obviously closely akin to a similar one observed in the pentatomid Brachystethus (Schrader, 1946).

The two sex chromosomes apparently are not affected by the anomalous behavior of the autosomes. They lie side by side in a compact and narrow spindle of normal length and undergo an orthodox equational division. In some cells the chromatids of the Y separate faster than those of the X and may precede them to the poles. As soon as the anaphase movement of the sex chromosomes is initiated, the autosomal aggregate once more approaches the polar axis, and by mid-anaphase is usually close to or even in contact with the sex chromosome spindle. This return also occurs in the equatorial plane of the cell, and is correlated with a shortening of the chromosomal fibers as well as the lengthening of the interpolar distance and cell as a whole—both of which will of course bring the autosomal aggregate closer to the polar axis again (Figs. 27 to 29).

Although the autosomes, aggregated as they are, pass through these maneuvers as a unit, there is evidence from the beginning that one of them plays a special role. Already at the first trace of division in the X and Y chromosomes, a single large chromosome protrudes from the autosomal clump, showing a well formed chromosomal fiber connection with one center and clearly oriented toward it. In such a position it appears more condensed than the rest of the autosomes, a condition which would be difficult to discern while it is still in the midst of the vacuolated, unevenly staining aggregate (Figs. 27 and 28). The reaction of this autosome to the pole is quite independent of the sex chromosomes, but it is obviously hindered in its movements—probably because of the "stickiness" that tends to hold all the autosomes together. As a result the two sex chromosomes are well on their way toward the poles before this autosome has disengaged itself from the encumbrance (Figs. 29 and 75).

Soon after it has left the aggregate, a second large autosome begins to dissociate itself from the rest of the autosomes. The extent to which it succeeds in this is highly variable in different cells, but in most cases it at least protrudes from the mass before the division is finished (Fig. 30). Often, while the first autosome is still fairly



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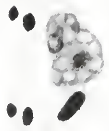

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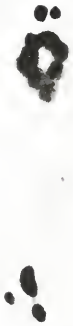

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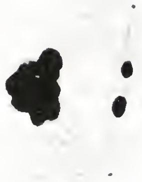

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Mecistorhinus melanoleucus—Harlequin Lobe

FIGURE 27. Early anaphase I; autosomal aggregate beginning to return (Gentian violet).
FIGURE 28. Mid-anaphase I; (Gentian violet).
FIGURE 29. Late anaphase I (Gentian violet).
FIGURE 30. Early telophase I (Gentian violet).
FIGURE 31. Late telophase I (Hematoxylin).
FIGURE 32. Early anaphase II—large cell (Gentian violet).
FIGURE 33. Mid-anaphase II—large cell (Hematoxylin).
FIGURE 34. Early telophase II—large cell (Hematoxylin).
FIGURE 35. Telophase II—large cell (Hematoxylin).

close to the aggregate, it shows a Feulgen-positive connecting thread with the second one, although this is usually severed very shortly (Fig. 29).

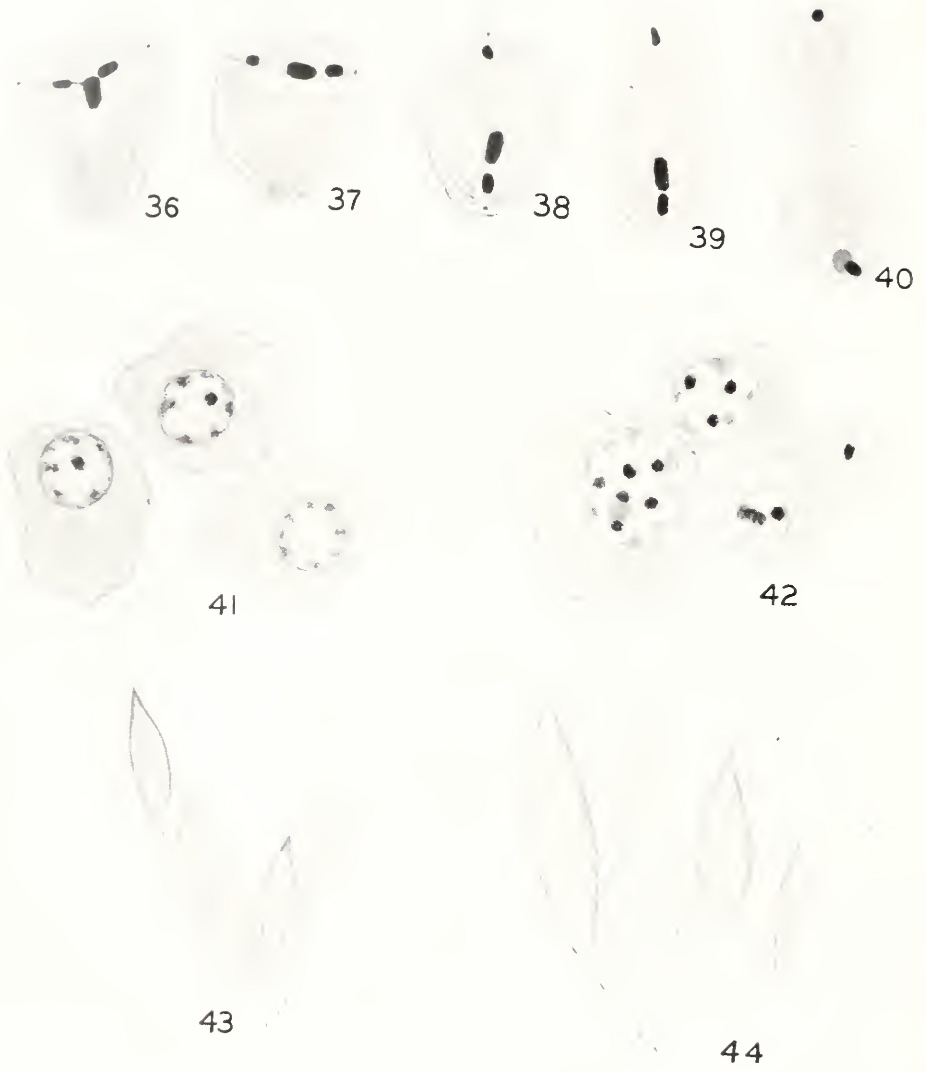
The rest of the autosomal clump, which is by now very close to the polar axis, also shows some response to the mitotic forces and frequently undergoes some elongation in the polar direction. Its effective movement however is always opposite in direction to that of the first autosome and hence it approaches the other pole. The second large autosome may sometimes almost reach the middle of the cell, but in only a single case has it proceeded so far that the ensuing cleavage constriction promises to include it in the same cell with the first autosome. Indeed, a count of 100 small second spermatocytes (those which do not receive the main part of the autosomal aggregate) has revealed no such accidental inclusion and it must be very rare. Further, in all observed cases (of which there are many dozens) this second autosome has rejoined the aggregate by the time that the second division is begun so that its mitotic motion must finally be reversed (Fig. 31). Possibly it initially follows the large autosome only because it is dragged along by the connecting thread. The final result of these maneuvers is that the large autosome and the rest of the autosomal aggregate always go to opposite poles. Since the former is the first to evince any reaction to the mitotic forces, one is almost forced to the hypothesis that it determines the direction of movement on the part of all the remaining autosomes.

These two autosomes that tend to disengage themselves from the aggregate are patently larger than any but the two largest chromosomes of the diploid set. At the same time they do not seem quite to reach the size of that large pair and it may therefore be that we are dealing with the two chromatids of only one of the latter. But admittedly the changes in the state of autosomal condensation during meiosis make it difficult to decide the matter on the basis of size alone. The later behavior of these exceptional chromosomes in the spermatids would however seem to support their identification as chromatids rather than whole chromosomes.

The second spermatocytes resulting from this first division, anomalous though it may be, are thus very constant in composition. Half of them contain the two sex chromosomes and only one autosome; the rest carry all the remaining autosomes as well as the sex chromosomes. The latter are much the larger cells of the two, as might be expected (Fig. 31).

The second division

Large cell: The chromosomes carried by these second spermatocytes comprise the sex chromosomes as well as all of the autosomes except the single one in the smaller cell. There is no interkinesis. In its general aspects the division of this cell simulates the first division. As the X and Y take their position on the new spindle, chromosomal fibers are also formed between the two poles and the autosomal aggregate. Almost simultaneously, the latter is shifted toward the side of the cell, the displacement being very similar to that observed in the first division (Figs. 32 and 76). However the aggregate now begins to be less spongy in appearance and there are other indications that its autosomal constituents are once more undergoing condensation (Fig. 33). During the anaphasic separation of the X and Y, the aggregate is again drawn toward the middle of the cell. It may be noted that when it reaches the compact little sex chromosome spindle, its surface of contact with it becomes smooth and concave, indicating that it is under some pressure (Fig. 33).



Mecistorhinus melanoleucus—Harlequin Lobe (except Figs. 41 and 43)

FIGURE 36. Beginning of Division II—small cell; large autosome connected with both X and Y (Hematoxylin).

FIGURE 37. Early anaphase II—small cell (Hematoxylin).

FIGURE 38. Late anaphase II—small cell (Hematoxylin).

FIGURE 39. Early telophase II—small cell (Hematoxylin).

FIGURE 40. Late telophase II—(Hematoxylin).

FIGURE 41. Early spermatids—normal lobe (Hematoxylin).

FIGURE 42. Early spermatids—harlequin lobe (Hematoxylin).

FIGURE 43. Late spermatids—normal lobe (Hematoxylin).

FIGURE 44. Late spermatids—harlequin lobe (Hematoxylin).

Then as the anaphase progresses and the cell and spindle elongate, the aggregate is drawn out between the two poles and many of its component autosomes are thus strung out in a roughly linear order (Figs. 34 and 78). But the tendency to stick and adhere to each other remains strong so that even when several individual chromosomes have been pulled out of the aggregate they usually still show thick, Feulgen-positive connections with each other and with the remaining aggregate (Figs. 34 and 35). The division so far as the autosomes are concerned is therefore obviously a haphazard one and the aggregate is frequently distributed very unevenly to the two spermatid cells.

The spermatids resulting from the division of the large second spermatocyte are therefore exceedingly variable in composition. Since the sex chromosomes segregate normally, all spermatids carry either an X or a Y, but the number of autosomes is largely a matter of chance. Nevertheless no cases have been observed in which the latter have all gone to one pole.

Small cell: As is the case with the large second spermatocytes there is no interkinesis of any kind and the final stages of the first division merge directly into the beginnings of the second. Indeed before the anaphase movement of the first division has been completed, the two daughter centrioles at each pole (each centriole appears double already at metaphase) have separated and begin their migration to establish the polar axis of the second division, at right angles to the first (Fig. 31). In some cells the sex chromosomes respond and orient to these new poles before they have completed the anaphasic movement. But apparently this precocious movement is then reversed, for in slightly later phases when the new axis has been established, the two sex chromosomes are near each other or in actual contact in the middle of the cell. Here too now lies the large autosome which has been delayed in its arrival at the pole. It may be in contact with neither, either or both sex chromosomes, or it may be connected with either or both through Feulgen-positive bridges (Fig. 36).

Chromosomal fibers are formed already before the three chromosomes have gathered at the midpoint, but so far as can be seen the tiny spindle is concerned only with the X and Y. No chromosomal fibers can with certainty be traced to the autosome. The sex chromosomes behave just as they do in a normal cell and after meeting in the middle of the spindle they separate to opposite poles in a regular segregation.

It is the behavior of the autosome that presents some puzzling aspects, as it already has done in the first division. The outstanding feature of this behavior lies in the fact that it nearly always goes to the same pole with the X. In so doing it appears to have little or no independent mitotic movement, acting merely as a satellite of the sex chromosome. Its dependence on the latter is shown not only by the absence or poor development of chromosomal fibers already mentioned, but is indicated also by its behavior before and during anaphase. In the grouping prior to the division, the autosome sometimes lies between the X and the adjacent pole, but when the anaphase movement is under way it always trails the sex chromosome (Figs. 36 to 40, and 77). Since during most late anaphases the autosome is attached more or less closely to the X, its behavior might be attributed to a simple adhesion between the two were it not for the fact that many earlier anaphases show no such connection. The latter thus frequently seem to be established after the mitotic movement is under way. Indeed, connections of this sort cannot be decisive in any case since prior to the division the autosome often lies in contact with both sex chromosome or shows a Feulgen-positive bridge to the Y and not to the X (Fig. 36). Evidently this is later broken so that the mitotic association with the X must involve some selective action not dependent on such physical bonds. Whatever the underlying mechanism may be, the results of the division admit of no doubts concerning the constancy of the relationship between these two chromosomes. In 100 clear side views of late anaphases there were only six that admitted the possibility of an association of the autosome with the Y. In two of these the autosome is clearly much closer to the Y than the X and probably going to the same pole, whereas in the remainder the identification of the X and Y is not certain. In close to 95 per cent of the cases therefore, the autosome accompanies the X to the pole and not the Y. Generally speaking then, the small spermatids which come from this division are of two types: one, which carries the X and the autosome; and another which contains only the tiny Y.

Spermatids and sperms

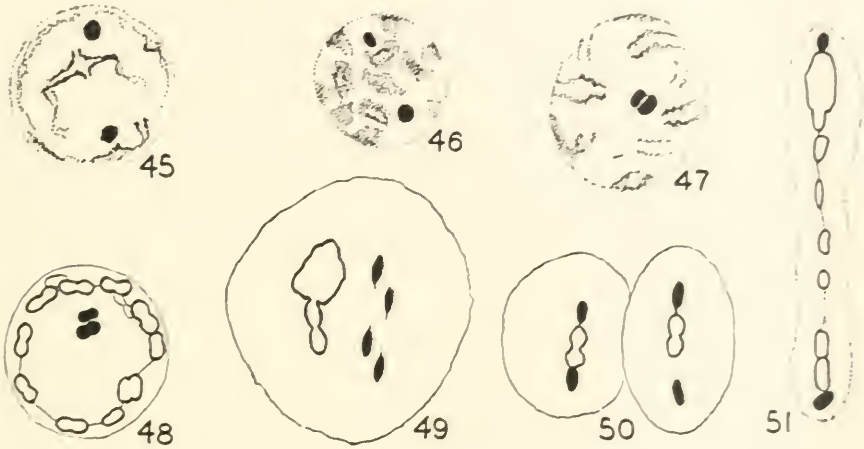
The two anomalous spermatocyte divisions of the harlequin lobe thus give rise to four main types of spermatids: X + one large autosome; Y; X + a variable number of autosomes; Y + a variable number of autosomes. Since in the spermatid the autosomes scatter again and tend to be distributed peripherally on the new nuclear membrane before becoming diffuse, rather dependable counts are often possible. Both of the small types of spermatids are readily recognizable and it is to be noted that the close association between the X and the autosome of the preceding telophase is maintained into an advanced spermatid stage (Fig. 42). In the large spermatids the number of chromosomes may be as low as five or six and frequently higher than twenty. The latter counts constitute conclusive evidence that the chromatids of the univalent autosomes have separated from each other, since the full, normal haploid number of the species is only seven (compare the normal spermatids of Fig. 41 with those of the harlequin lobe in Fig. 42). Since the autosome that is associated with an X in one of the small spermatids undergoes no such separation into smaller units, there is strong presumptive evidence (to support that which was adduced earlier) that it represents a chromatid which has already separated from its sister chromatid—the second large autosome of the first division.

Formation of sperms seems to proceed normally in all cells until the stage when the elongation nucleus shows the pointed apex which indicates the presence of the acrosome (compare Fig. 43 with Fig. 44). At about this time, when the chromosomes have become very diffuse and the contents of the sperm head seem structureless, the small types of sperms gradually stain more and more faintly. It finally becomes impossible to recognize them, whereas the larger types assume the attenuated, intensely stained form of the head that is also encountered in the normal lobes. It is more than probable therefore that the smaller sperms never attain maturity, whereas the large ones appear normal in every respect but size. They enter the sperm duct in a perfectly regular manner and there mingle with the sperms of the normal lobes.

MECISTORHINUS TRIPTERUS

The main points in which *Mecistorhinus tripterus* differs from *Mecistorhinus melanoleucus* involve the heteropycnosis of the autosomes and the behavior of the sex chromosomes. So far as the diploid complement of chromosomes is concerned, there is no perceptible difference, both having an outstandingly large pair of autosomes and a Y that is the smallest of all the chromosomes. However, already in the spermatogonial resting phases there is a less extensive heteropycnosis in *Mecistorhinus tripterus*, for then as well as later there are never more than two heteropycnotic bodies (instead of from three to five). The spermatogenesis in the normal lobes is entirely orthodox. The heteropycnosis in the large autosomes is here lacking entirely and hence there is no association between them and the sex chromosomes (Fig. 45).

The lack of autosomal heteropycnosis obtains also in the harlequin lobe (Figs. 46 and 47). As a consequence, when the equatorial ring is formed the two sex chromosomes lie in the approximate middle of the nuclear space instead of being carried to the periphery by the large autosomes (Fig. 48). The X and Y are at this time still in more or less intimate contact with each other and remain so until the first anaphase has begun.



Mecistorhinus tripterus—Harlequin Lobe (except Fig. 45)

FIGURE 45. Normal diakinesis; X and Y independent of autosomes (Feulgen).
FIGURE 46. Early diplotene stage (Feulgen).
FIGURE 47. Diakinesis; X and Y joined (Feulgen).
FIGURE 48. Equatorial ring in formation; X and Y independent of autosomes (Feulgen).
FIGURE 49. Anaphase I (Feulgen).
FIGURE 50. Anaphase II—small cell (Feulgen).
FIGURE 51. Anaphase II—large cell (Feulgen).

Another difference lies in the size relations between the X and the Y. In *Mecistorhinus melanoleucus* the X is markedly larger than the Y, but this difference is less pronounced in *Mecistorhinus tripterus*. In the latter also, as in most other pentatomids, the sex chromosomes become less sharply outlined in the second division and therefore it then becomes difficult at times to distinguish between them (Fig. 50). As a consequence, in about 25 per cent of fifty cells it is not possible to decide whether the autosome follows the X and there is at least the possibility that in such instances it accompanies the Y. Although there is thus no question that in this race too the association is between the X and the autosome in the great majority of cells, the case is not as clear-cut as it is in *Mecistorhinus melanoleucus*.

Mention should also be made of the fact that already in the first division, the first large autosome usually shows a median furrow (Fig. 49). If, as in *Mecistorhinus melanoleucus* this chromosome represents only one chromatid of one of the large univalents, the furrow is equivalent to a tertiary split. Whether that be correct or not, this split is not consummated even in the early spermatid, where the large autosome still maintains this appearance.

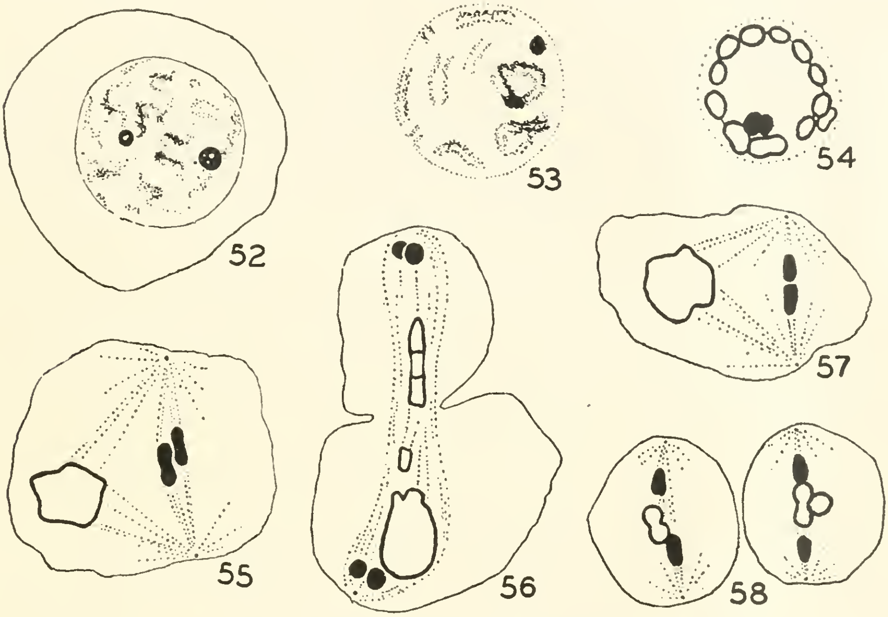
But in essence, the meiotic divisions of the two species are very much alike (Figs. 49–51, and 78). The difference in heteropycnosis does not affect the results and *Mecistorhinus tripterus* merely furnishes less decisive evidence anent the association between the X and the large autosome.

MECISTORHINUS SEPULCRALIS

The rather extensive material of this species from Brazil conforms closely in its general cytology to the Central American Mecistorhinus species. The diploid set is characterized by a very large pair of autosomes and the Y is again the smallest member of the set. The meiosis in the normal lobes follows an orthodox course although there are indications of heteropycnosis in a very restricted region of the two large autosomes.

This heteropycnosis is very much alike in normal and harlequin lobes. The spermatogonial resting phases may show as many as three or four very small heteropycnotic regions but more often only one larger one. In the meiotic prophase following the leptotene stage and up to diakinesis, it is difficult to recognize any heteropycnosis in the autosomes whereas both sex chromosomes are, as usual, heteropycnotic throughout. In the confused stage there are generally two heteropycnotic bodies, but whether these represent only the sex chromosomes or also certain autosomal regions it is not possible to say (Fig. 52). Only in mid-diakinesis do conditions allow a closer analysis. In the harlequin lobe, where we are dealing with univalents, both large autosomes then show heteropycnotic terminal regions, and these are joined so as to form closed rings as in *Mecistorhinus melanoleucus* (Fig. 53). Apparently this heteropycnosis is less extensive in the present species and this may account for the fact that the mutual attraction of such regions does not bring the two large univalents together in the "figure eight" formations that occur so often in the other forms. Probably for the same reason the association between the sex chromosomes and these autosomes is highly variable. Thus in some cells only one sex chromosome is joined to the heteropycnotic region of one univalent autosome while the other sex chromosome is free (Fig. 53). In late diakinesis such a condition is rare because, as in *Mecistorhinus tripterus,* there is then a strong tendency for the X and Y to come together. Hence the free sex chromosome often joins its attached mate and as a result both are carried into the equatorial ring of autosomes, just prior to the breakdown of the nuclear membrane (Fig. 54). In other cases the sex chromosomes may be entirely free and will then take a more or less central position in the nucleus at the time of the equatorial orientation of the autosomes.

But these various maneuvers that involve heteropycnotic attraction do not affect the course of the actual divisions in the harlequin lobe any more than in the normal lobes. In the former the sex chromosomes at both metaphases become detached from the autosomal aggregate, which then is displaced toward the side of the cell just as in *Mecistorhinus tripterus* (Figs. 55 and 57). In *Mecistorhinus sepulcralis* however, the first anaphase shows a definitely greater tendency for other chromosomes to follow the first large autosome out of the aggregate. Although the second large autosome is included as rarely in the smaller second spermatocyte as in the preceding species, a smaller autosome sometimes succeeds in following the first autosome into this cell (in four out of forty cells) (Fig. 56). Apparently both these autosomes retain the "sticky" condition of the earlier aggregate, for when they reach the pole the smaller joins the larger one and adheres to it in almost any position in random fashion. In the second division, both follow the X to the pole in the majority of cases (Fig. 58).



Mecistorhinus sepulcralis—Harlequin Lobe

Figure 52. Confused stage (Feulgen).
Figure 53. Diakinesis stage (Feulgen).
Figure 54. Equatorial ring; X and Y associated with large autosomes (Feulgen).
Figure 55. Metaphase I (Hematoxylin).
Figure 56. Late anaphase I (Hematoxylin).
Figure 57. Metaphase II—large cell (Hematoxylin).
Figure 58. Anaphase II—small cell (Hematoxylin).

But here too, as in the second division of *Mecistorhinus tripterus* the X and Y often show only a small size difference In perfect side views they can nearly always be distinguished, but if the cell is viewed at a slight angle it at once becomes difficult to do so. Hence, though *Mecistorhinus sepulcralis* also shows a definite preferential association between the X and the large autosome (as well as the smaller, if present) the possible occurrence of exceptions to the rule can no more be excluded than in the preceding form.

The division of the aggregate in the larger second spermatocyte occurs very much as it does in *Mecistorhinus tripterus.* The four main types of spermatids are therefore alike in all three species, comprising X + one autosome; Y; X + variable number of autosomes; Y + variable number of autosomes. The few exceptions in the present species are due to the occasional inclusion of a smaller autosome in the first named spermatid.

NEODINE MACRASPIS

This representative of another genus is in its cytological features almost a duplicate of *Mecistorhinus tripterus.* The cells and chromosomes of the harlequin lobe are slightly larger than those of the preceding forms. The large pair of autosomes however does not stand out quite so strikingly in its size as in the species of Mecistorhinus (Fig. 59). The normal meiosis is entirely orthodox (Figs. 60 to 63) and there is no heteropycnosis other than that of the sex chromosomes to complicate the prophases.

The harlequin lobe likewise lacks all autosomal heteropycnosis. The two sex chromosomes come into contact with each other during the confused period and this loose union is maintained through the diakinesis (Figs. 64 to 66) into the first metaphase (Fig. 67). They are entirely independent of the autosomes, and when the latter are marshalled to form their equatorial ring the X and Y are always found together in the middle of the nucleus (Fig. 66).

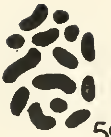
In the course of the first division, only one large autosome and no others join the X in the formation of the smaller second spermatocyte. This autosome may or may not exhibit the median tertiary split (Fig. 70).

The second division conforms to the general scheme described for the other species. The size difference between the X and Y is not as clear cut as in *Mecistorhinus melanoleucus*, but there is no doubt that in the great majority of cells, it is the X with which the single autosome is associated and not the Y (Figs. 70 and 71). The larger second spermatocyte divides irregularly and in the same manner as does the corresponding cell in Mecistorhinus.

In all essentials, therefore the meiosis in the harlequin lobe of Neodine parallels that of Mecistorhinus.

PLATYCARENUS NOTULATUS

In view of the uniformity in the occurrence of normal and harlequin lobes of the four preceding species, it comes somewhat as a surprise that in another species, *Platycarenus notulatus*, the conditions are entirely different. The structure of the testis departs radically from that of the other forms, there being only four instead of seven lobes, with a more compact instead of a serial arrangement. The diploid set of fourteen chromosomes is marked by no strikingly large pair of autosomes and only the exceptionally small size of the Y merits any notice. There is no harlequin lobe and the spermatogenesis takes a very orthodox course throughout the testis. The case thus serves warning that the cytological conditions are by no means uniform in the tribe Discocephalini as it is now constituted by the systematists.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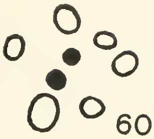
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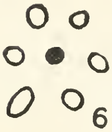
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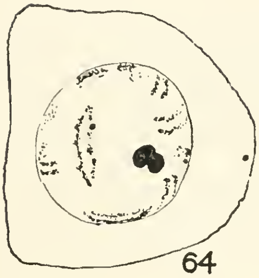
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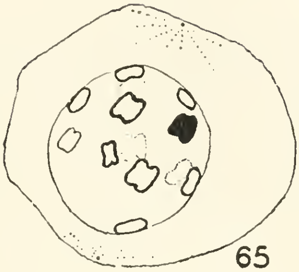
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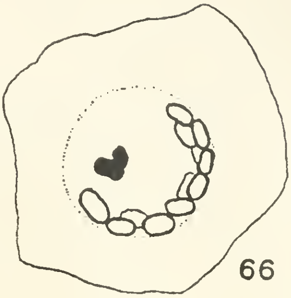
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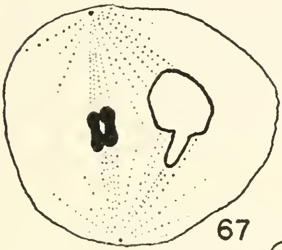
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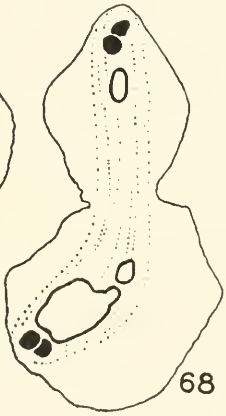
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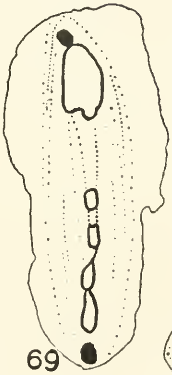
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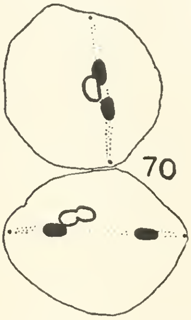
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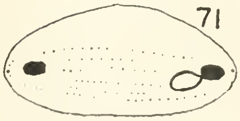
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Neodine macraspis

Normal Lobe:

- Figure 59. Spermatogonial metaphase (Hematoxylin).
- Figure 60. Metaphase I (Hematoxylin).
- Figure 61. Metaphase II (Hematoxylin).
- Figure 62. Telophase II; polar view with Y (Hematoxylin).
- Figure 63. Telophase II; polar view with X (Hematoxylin).

Harlequin Lobe:

- Figure 64. Early diakinesis; joined X and Y independent of autosomes (Hematoxylin).
- Figure 65. Late diakinesis; 12 univalents + joined XY (Hematoxylin).
- Figure 66. Formation of aggregate; joined X and Y separate (Hematoxylin).
- Figure 67. Early anaphase I (Hematoxylin).
- Figure 68. Telophase I (Hematoxylin).
- Figure 69. Telophase II—large cell (Hematoxylin).
- Figure 70. Anaphase II—small cell (Hematoxylin).
- Figure 71. Telophase II—small cell (Hematoxylin).

Discussion

The most striking anomalies of the meiosis in the harlequin lobe of the Discocephalini may be summed up as follows: 1) Omission of pairing, 2) Clumping or aggregation of autosomes, 3) Preferential association between the X chromosome and a certain autosome.

Omission of pairing

The failure of the leptotene homologues to come together in synapsis has at present no cytological explanation. The only indication that conditions are not entirely orthodox at an earlier stage lies in the fact that the leptotene threads are less fine and attenuated than they are in normal lobes. In short they are not despiralized to the same extent as chromosomes that undergo the characteristic pairing reaction.

The further prophase behavior of the unpaired chromosomes is however of great interest. These univalents pass through all the meiotic phases that distinguish bivalents from the chromosomes of non-meiotic mitoses. They rapidly condense after the leptotene stage and a diplotene split makes its appearance; there follows a regular confused period; and the latter is in turn succeeded by an entirely typical series of diakinetic maneuvers. It would seem therefore that these characteristic prophase manifestations of meiosis are not at all conditioned by the phenomenon of pairing and, given certain basic conditions, the chromosomes run through the gamut of meiotic changes even when this most obvious feature of the meiotic prophase has been omitted.

Clumping and displacement of autosomes

The clumping or aggregation of autosomes which occurs suddenly just before the first metaphase and is not completely abandoned until the spermatid stage, is one of the most striking features of the harlequin lobe. It is only natural to seek some interrelation between this anomaly and the failure of synapsis that precedes it, but the connection is certainly not a direct one. Thus the omission of pairing in Loxa (Schrader, 1945b) is followed by no clumping in the first division (although there are indications of "stickiness" in the second). The most decisive evidence against such an interrelation is furnished by Brachystethus (Schrader, 1946). There the clumping of autosomes is even more persistent than in the Discocephalini but an omission of pairing obviously cannot be held responsible since the autosomes undergo normal synapsis and form typical tetrads.

The occurrence of autosomal heteropycnosis in the prophase of *Mecistorhinus melanoleucus* can also not be held responsible for the later aggregation of autosomes. In *Mecistorhinus tripterus* the clumping is just as marked as in the first named species, but there is no heteropycnosis in the autosomes at all. Indeed, despite the peculiar maneuvers that such heteropycnosis brings about especially during diakinesis, the meiotic divisions themselves are not perceptibly affected. They are essentially the same, whether autosomal heteropycnosis be present or not.

In all these considerations of the clumping phenomenon it must be remembered that the mitotic apparatus remains normal and cannot be held directly responsible for the anomalies. The justification for this conclusion lies not only in the fact that chromosomal spindle fibers are regularly formed between the centrioles and the clumped autosomes, but also in the perfectly normal spindle relations of the two sex chromosomes.

It is the orthodox behavior of the sex chromosomes that may furnish a clue to the autosomal behavior. As usual, both the X and the Y chromosome are persistently heteropycnotic and at first metaphase are fully condensed. The autosomes however depart from their usual cycle of condensation and diffuseness. Already at prometaphase their precocious reaction to the polar forces would suggest a condition at variance with that which normally obtains—perhaps a more advanced state of condensation. Such abnormal timing is indicated more directly when the disintegration of the nuclear membrane finally occurs. The autosomes have then begun to reverse the normal sequence of development and when they undergo the clumping reaction they are in process of returning to the diffuse condition rather than attaining their final condensation. It is this untimely regression in nucleination (for there appears to be only a slight uncoiling) that probably underlies their clumping.

Such a hypothesis implies a close correlation between the state of condensation and the mutual reactions of chromosomes. It may be objected that during a normal prophase, when the chromosomes pass through all stages of condensation, no such clumping ever occurs. But it must be remembered that during the entire prophase a nuclear membrane is present and there can now be little doubt that this exerts the most far-reaching influence on chromosomal behavior (Schrader, 1941). Indeed when the membrane finally breaks down there is in normal cells also an immediate clumping of chromosomes which do not separate again until they reach final condensation shortly thereafter. And in the normal telophase, when the chromosomes have started to return to the diffuse condition, there is again a tendency to clump. It is only after the new nuclear membrane has been formed that the chromosomes separate and scatter once more. The so-called mutual repulsion of the chromosomes in these Pentatomidae and perhaps other forms as well thus appears to occur only when they are fully condensed or else are contained within a nuclear membrane.

It is possible that here also lies the key to the extrusion of the autosomal aggregate from the midregion of the spindle. As has already been said, the orthodox behavior of the sex chromosomes in the same spindle proves that it is not in the spindle apparatus that an explanation is to be found. It is the autosomes and kinetochores that must be held responsible and the abnormality in their condition which results in clumping is probably also involved in their lateral displacement. That this aberrant condition is only temporary is indicated by the fact that there is a partial return to normal behavior in the second division. The aggregate of autosomes is then stretched between the two poles and, though still "sticky," many of its components become dissociated from each under the strain and again appear as individual chromosomes. By the time the spermatid stage has been reached their behavior appears to be normal and they scatter over the periphery of the nuclear membrane quite like the chromosomes of the neighboring lobes.

It is safe to assume that the establishment of the metaphase involves a set of precisely adjusted interactions between centers, chromosomes and kinetochores. That these interactions involve nothing more than a system of repulsions would seem to be very unlikely, and indeed the retention of the chromosomes within the compass of the equatorial plate almost demands forces of a positive nature. Whether these be contractile chromosomal spindle fibers or a zone of actual attraction in the equator or both, it is quite possible that an upset in the condition of one of the elements involved might allow the forces of repulsion to gain the ascendancy.[2] On such a basis the regressive nucleination that is perhaps accompanied by a partial weakening of the kinetochores may well account for the expulsion of the autosomes in these Discocephalini.

The preferential segregation of one autosome

The special behavior of one of the autosomes and its association with the X chromosome has already been described. In analyzing its movements the following factors should be considered:

In the first division this chromosome shows no connection with either of the two sex chromosomes and its mitotic reactions are directed solely to the nearest pole. Its only distinction from the rest of the autosomes lies in a slightly greater condensation which is not very pronounced but may explain why it behaves more normally than they in this division. In the division of the smaller second spermatocyte this independence of the autosome is lost and to all intents and purposes it then becomes a satellite of the X chromosome.

The available evidence suggests that this chromosome represents a single chromatid of one of the large, univalent autosomes. It is probable that the two chromatids of this large univalent separate in a normal mitotic movement and that position in the aggregate determines which one frees itself and joins the two sex chromosomes at one pole. Its mate always moves to the opposite pole and with it goes the rest of the aggregate. The complications that attend these maneuvers arise primarily from the stickiness that tends to hold all autosomes together.

This provisional explanation of the peculiarities of the first division does not however throw much light on the division of the smaller second spermatocyte. The preferential segregation of the autosome is obviously a consequence of some interrelation with the X chromosome. To be sure, the two chromosomes are often connected by a Feulgen-positive strand or direct adhesion to each other. But before anaphase such a connection is sometimes also established between the autosome and the Y, and in still other cases there is no visible connection with either sex chromosome. Nevertheless, in the great majority of cases (about 95 per cent in *Mecistorhinus melanoleucus*) it is the X with which the autosome is finally associated at telophase and not the Y. The evidence as it stands therefore admits of only one interpretation which has only a few parallels in the literature—the preferential segregation of the autosome is due to some kind of attraction between it and the X (or, much more unlikely, its repulsion by the Y). A directed movement is involved in any case and since the X behaves just as it would in a perfectly normal cell it is the autosome itself which is primarily accountable.

Evolutionary aspects

It is a matter of surprise that so striking a feature as is comprised by the harlequin lobe should have escaped the notice of cytologists for so many years. To be sure it has so far been encountered only in the hemipteran family of Pentatomidae, but that particular group of insects has been subjected to more intense cytological study than almost any other comparable group except the orthopteran family of

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[2] In a short article that has just come to hand, Östergren (Bot. Notiser, 1945) suggests that if the spindle is a tactoid such an interplay of forces may well be involved.

Acrididae. Work on some 70 species has been published and among the investigators have been such outstanding cytologists as Montgomery, Wilson, and Geitler. To now discover harlequin lobes in some eight genera and eleven species, widely scattered through the family, can hardly mean that their occurrence has simply been overlooked hitherto. We must seek an explanation elsewhere and in such a quest one cannot fail to be struck by one aspect of the situation. It so happens that despite the great number of species investigated all cytological work has been confined to Pentatomidae from the Palaearctic and Nearctic regions. The rich pentatomid fauna of the Oriental, Australasian, and Ethiopian regions has never been touched by cytologists, and only recently have Neotropical species been investigated. It is among the latter that I have encountered all the species that carry harlequin lobes and the question might well be asked whether the occurrence is not correlated with a tropical habitat. But that is not to say that all tropical Pentatomidae are characterized by such lobes for my own investigations have shown that a number of species of such genera as Alcaeorrhynchus, Arocera, Edessa, etc., do not possess it. Nevertheless the correlation is sufficiently striking to make an inquiry into the effects of various factors in tropical conditions on the development of reproductive organs well worth while.

The occurrence of harlequin lobes in four different species of the tribe Discocephalini collected in localities as far apart as Costa Rica and southern Brazil (about 5400 km. or 3350 miles) would seem to justify the conclusion that such a feature was present in the ancestral species of the tribe. Its absence in a fifth, a species of Platycarenus, might be attributable to loss in the course of evolution. But harlequin lobes are found also in some members of entirely different tribes such as the Halyini (unpublished) and the Pentatomini, and in such a remote form as Brachystethus (Schrader, 1946) its cytological character is obviously very similar to that of the Discocephalini. On the basis of our original reasoning we would thus arrive at the conclusion that a harlequin lobe characterized not only the first discocephalinid but was present already in the more remote ancestor of the whole family of Pentatomidae. Its sporadic occurrence at the present time may therefore be representative of evolutionary survivals that are possible only under certain conditions, though of course it is possible that the genetic basis for the harlequin lobe has existed for a long time but that this can express itself only under certain rather limited conditions. In either case, the deciding factor may well lie in the environment which happens to be more often favorable in tropical than in temperate regions.

Since the sperms of the harlequin lobe are ordinarily nonfunctional in the hereditary sense, it is obvious that in such species there is a very great loss of gametes. Natural selection might confidently be expected to eliminate so wasteful a development unless it also confers certain advantages that compensate for the loss of functional sperms. It has been suggested (Schrader, 1946) that these advantages may lie in the nucleoproteins that are carried into the eggs by supernumerary sperms. Polyspermy is of extremely common occurrence in the fertilization of insect eggs and it is probable that the nucleoproteins introduced by the extra sperms are utilized by the growing embryo. The sperms from the harlequin lobe are usually much larger than normal sperms and hence would contribute proportionately larger amounts of nuceloproteins. Species with harlequin lobes may therefore hold an advantage over those that lack them—an advantage that takes the form of an increase in materials that are of special value for the developing embryo. Certain it is that so prosperous a species as *Mecistorhinus tripterus* which has a distribution from Vera Cruz, Mexico, to São Paulo, Brazil, has found the possession of a harlequin lobe no disadvantage.

The sporadic and yet widespread occurrence of harlequin lobes among the species of Pentatomidae argues strongly that we are dealing with a basic condition. The problem of why a certain lobe in the testis always takes a special course in its development may in the end be no different from that involved in such a case as the development of diverse types of digestive glands from the embryonic gut. In other words, we are confronted here, as well as there, by the old question of differentiation.

SUMMARY

1. In the testes of four species of the pentatomid tribe Discocephalini, the fifth lobe always shows a special type of meiosis.

2. There is no pairing in this lobe.

3. Just prior to metaphase I all the autosomes aggregate in one clump. The sex chromosomes behave quite normally through both meiotic divisions.

4. In the first division the X and Y divide equationally as in normal cells. One large autosome becomes dissociated from the aggregate and reaches the pole opposite to that attained by the rest of the aggregate.

5. A large and a small second spermatocyte result from this first division. In the small one, the single autosome goes to the same pole as the X in the great majority of second divisions.

6. In the division of the larger second spermatocyte, the aggregate is irregularly pulled into two groups.

7. Four main types of spermatids result: X + variable number of autosomes; Y + variable number of autosomes; X + one large autosome; Y. The last two types probably never reach the mature sperm stage.

8. It is pointed out that so firmly established a feature as this seemingly wasteful development in the fith or "harlequin" lobe must have some compensatory advantage, and its cytological and evolutionary significance are discussed.

All figures are photographs from harlequin lobe; Figs. 72 to 77 inclusive from *Mecistorhinus melanoleucus;* Fig. 78 from *Mecistorhinus tripterus.*

FIGURE 72. Formation of equatorial ring (Hematoxylin).

FIGURE 73. Early metaphase I with the two sex chromosomes close together in the middle and the autosomal aggregate displaced to one side (Hematoxylin). Shown in two of the cells.

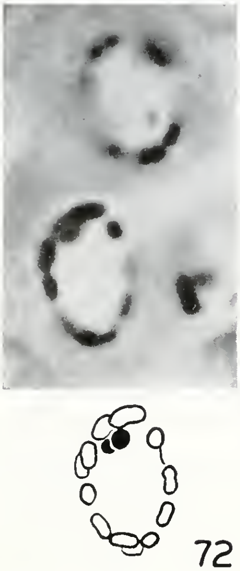
FIGURE 74. Beginning of anaphase I; X and Y in middle, large autosome beginning to protrude from displaced aggregate (Gentian violet).

FIGURE 75. Late anaphase I, photographed at two different levels; large autosome leaving aggregate, and an X and Y approaching each pole (Gentian violet).

FIGURE 76. Anaphase II (large cell); aggregate displaced to one side and X and Y separating to opposite poles (X to lower pole) (Hematoxylin).

FIGURE 77. Anaphase II (small cell); X with autosome going to upper pole and Y to lower pole (Hematoxylin).

FIGURE 78. Telophase II (large cell); aggregate becoming dissociated between separating X (lower pole) and Y (upper pole) (Feulgen).



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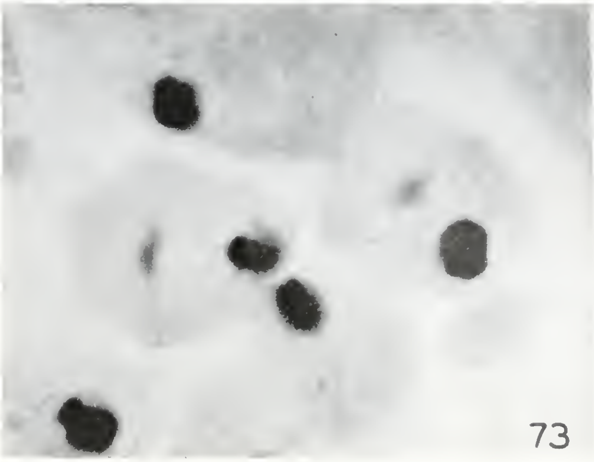

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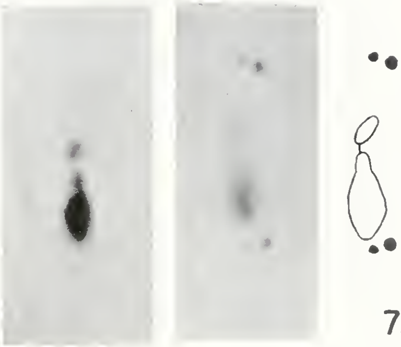

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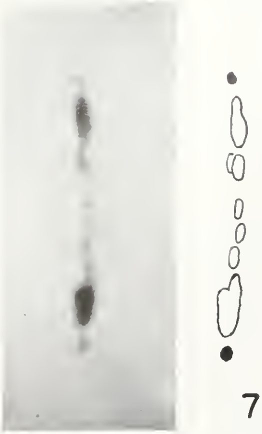

78

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THE EFFECT OF THE ADULT ANTERIOR PITUITARY HORMONE ON THE TADPOLES AND THE IMMATURE MALE FROGS OF THE BULLFROG, RANA CATESBIANA

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Introduction

The interrelationship of the anterior pituitary hormone and the gonads of vertebrates has been well established. There is evidence that the amphibian gonads, under the influence of the anterior pituitary hormone stimulation, will either respond immediately (in mature animals) by the liberation of mature gametes or will differentiate (if immature) in much the same manner as they do during the breeding season, with the exception that these artificially stimulated changes are accomplished at a much faster rate. This statement is supported by the work of Riddle and Flemion (1928) on the dove; Houssay and Lascano-Gonzalez (1929) on the South American toad; Herre and Raiviel (1939) on larvae of Triton; Burns (1930) and Burns and Buyse (1934) on Amblystoma; Evans (1935) on the lizard; Forbes (1937) on the alligator; Rugh (1937, 1939, and 1941) on frogs of different genera; Puckett (1939) on the bullfrog tadpole, and Glass and Rugh (1944) on the mature frog, *Rana pipiens*. In this latter paper it was shown that while there are seasonal changes in the frog testis, indicating cyclical maturation, the gonad will respond at any time of the year to frog anterior pituitary hormone stimulation by the liberation of any mature spermatozoa which are present, and that frequently (especially during active maturation of August) the seminiferous tubules will also contain other maturation stages. Recently Schreiber and Rugh (1945) have studied the response of the testes of recently metamorphosed *Rana pipiens* to the pituitary hormone and to estradiol-benzoate.

It was the purpose of this investigation to determine the effect of the anterior pituitary hormone of the adult frog on the gonad of the pre- and the post-metamorphosing male bullfrog. The testis can be differentiated from the ovary macroscopically even before tail absorption in the tadpole of *Rana catesbiana*.

Materials and Methods

The tadpoles of *Rana catesbiana* were collected in the vicinity of Woods Hole, Mass., and were used immediately. During the month of August all stages of pre- and post-metamorphosis are available in great abundance. These tadpoles are in their second year of growth, and generally go through the stages of metamorphosis rather rapidly, depending upon the water temperature and the amount of food available. Since these tadpoles were well fed in their natural habitat, there was no attempt to feed them during the short period of the experiment, three to four days.

Tadpoles were selected in five stages:

- *a.* Pre-metamorphic stage where there were four well-developed appendages and a full-length tail fin.
- *b.* Post-metamorphic stage where the body length was less than 45 mm. and the tail had just been absorbed, but could be identified as a stub. These frogs had just shifted to lung breathing.
- *c.* Frogs of body length of 65 mm.
- *d.* Frogs of body length of 95 mm.
- *e.* Mature, full-grown frogs whose body length was at least 120 mm.

These five stages represented a graded series from pre-metamorphosis to the sexually mature adult male, covering a period of normal development ranging from August of one year to the summer of the following year, at least.

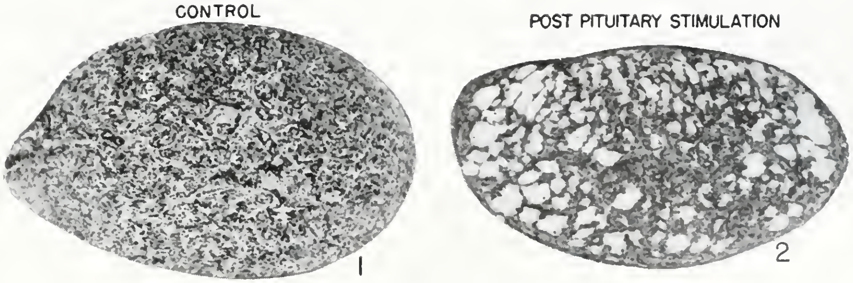
The experimental animals were injected within a few hours or, at the most, within 24 hours of collection in the ponds. In most cases the right testis was removed and fixed and the animal was allowed to recover from the gonadectomy and the abdominal wound was permitted to heal for 24 hours. Then each animal was injected with two whole anterior pituitary glands from sexually mature, pre-ovulating female bullfrogs. This is a dose known to be adequate to induce sexual activity in adults (Rugh, 1943). The hormone was injected whole, through a large bore hypodermic needle, into the body cavity, along with about 2 cc. of distilled water. The animal was then placed in a battery jar with a small amount of water and kept at laboratory temperatures. Forty-eight hours later each animal was sacrificed and the remaining (left) testis was removed and fixed in a manner identical with that for the right testis previously removed. In all instances the gonad was removed without direct handling for it has been shown (Rugh, 1939; 1941) that mere manipulation of the living testis will often alter the internal picture. The fat bodies were used as "handles" in excision and removal of the testis. The whole organ was placed in Bouin's solution for 24 hours; slowly run up through to 70 per cent alcohol where the yellow picric stain was removed by the addition of 5 per cent by volume of concentrated NH_4OH for several half-hour changes; dehydration was completed in higher alcohols; clearing in xylol; embedding in a mixture of 90 per cent paraffin (58° MP), 5 per cent bayberry wax, and 5 per cent beeswax. The gonads were sectioned at 8 microns and were stained with Heidenhain's iron-haematoxylin with counterstain.

The photographs were taken on Microcopy film (35 mm.) by means of a Mifilmca adapter.

OBSERVATIONS AND EXPERIMENTAL DATA

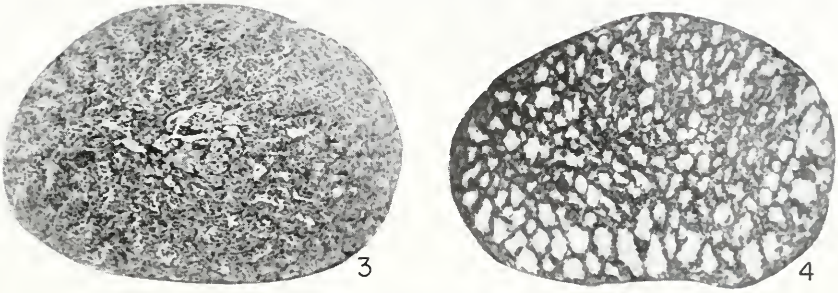
Pre-metamorphic stage

The testis of the pre-metamorphic bullfrog tadpole, which stage possesses all four appendages and a tail, appears as a solid yellowish organ associated with the adjacent reddish kidney by means of the double-layered mesorchium. In section (Figs. 1 and 9) the gonad does not show well-formed seminiferous tubules partly because there is an abundance of interstitial tissue and there are no late maturation stages and no lumina. Scattered around the periphery of the developing seminiferous tubules are found huge primary spermatocytes which seem to be adherent to the base-

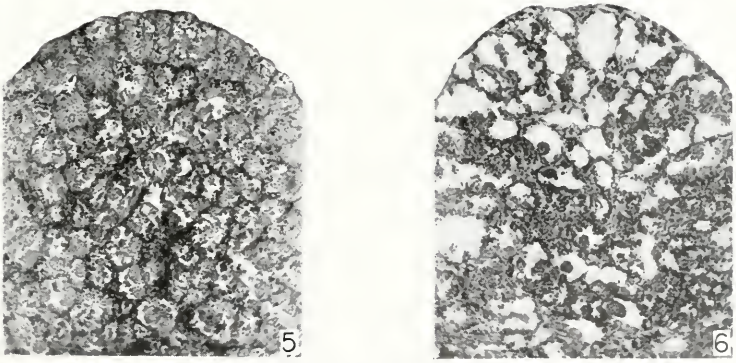

CONTROL

POST PITUITARY STIMULATION

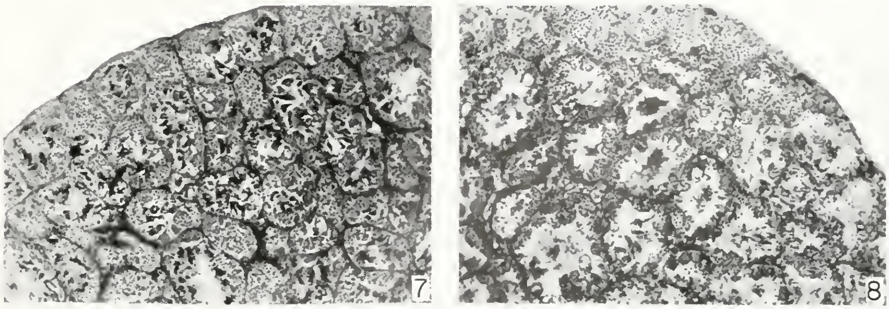
PRE - METAMORPHOSIS



POST - METAMORPHOSIS



65 MILLIMETER BODY LENGTH



95 MILLIMETER BODY LENGTH

PLATE I. Effect of anterior pituitary hormone on testis of immature bullfrog, *R. catesbiana*.

ment membrane of the tubule. There are also oval-shaped darkly staining nuclei of the spermatogonia, and occasionally there may be seen (toward the region of the future lumen) some secondary spermatocytes. The average length of these testes is 2.18 mm. and the average diameter is 0.80 mm.

When the pre-metamorphic tadpole is subjected to the anterior pituitary hormone (by coelomic injection) the most obvious change in the testis is the vacuolization of the forming seminiferous tubules (Figs. 2 and 10) so that the entire gonad takes on a sponge-like appearance. The peripheral region of the gonad is always the first to be affected, and the most vacuolated. Under high power magnification it is noted that the only cells remaining within the seminiferous tubules are those attached to or an integral part of the basement membrane. The other cells which had previously occluded the lumen will be seen in the collecting tubules and the vasa efferentia, indicating that they were enroute through the reproductive tract at the time the gonad was fixed. One gets the impression that the gonad was flushed out of all loosely attached cellular components.

The size variations of the control and the experimental gonads were not appreciably different.

Post-metamorphic stage, body length less than 45 mm.

In this stage the gonad itself has enlarged considerably so that the maximum length of the testis is about 2.6 mm. and the maximum diameter is about 1.8 mm. The shape of the gonad is also changed, since its length increased about 40 per cent while its diameter increased about 125 per cent.

In the control testis (Figs. 3 and 11) there is some evidence of the forming seminiferous tubules, and maturation stages from spermatogonia to mature spermatozoa are seen. This indicates that coincidental with the drastic changes of metamorphosis the bullfrog tadpole's testis first acquires structurally mature spermatozoa. Whether or not these spermatozoa are functional has not yet been determined. Clusters of cells are seen, all in the same stage of maturation, with the progressively advanced stages more nearly in the center of the seminiferous tubules.

Here again, when the testis is subjected to the anterior pituitary hormone of the adult, there is a liberation from the tubules of all freely associated cells (Figs. 4 and 12) so that the post-stimulated gonad has much the same appearance as that of the experimental testes of the pre-metamorphic stage (Figs. 2 and 10). There remain only the spermatogonia, primary spermatocytes, and the few interstitial cells. The majority of cells found within the collecting tubules and the vasa efferentia (Fig. 17) are secondary spermatocytes.

65 mm. body length stage

By this stage of development the testis has acquired its adult proportions and measures about 3.35 mm. in length and 2.0 mm. in diameter. The surface has become lobulated due to the further development of the seminiferous tubules about the periphery. Nests of maturing cells are seen (Figs. 5 and 13) representing all stages from the primary spermatocytes to the spermatids and mature spermatozoa. The early maturation figures are in great abundance with the pachytene groups the most prominent (Fig. 13). It is at this stage that the cysts or nesting groups of cells become distinct from each other, demarking those clusters of cells, all of which originate from the same spermatogonium. With the enlargement of the seminiferous

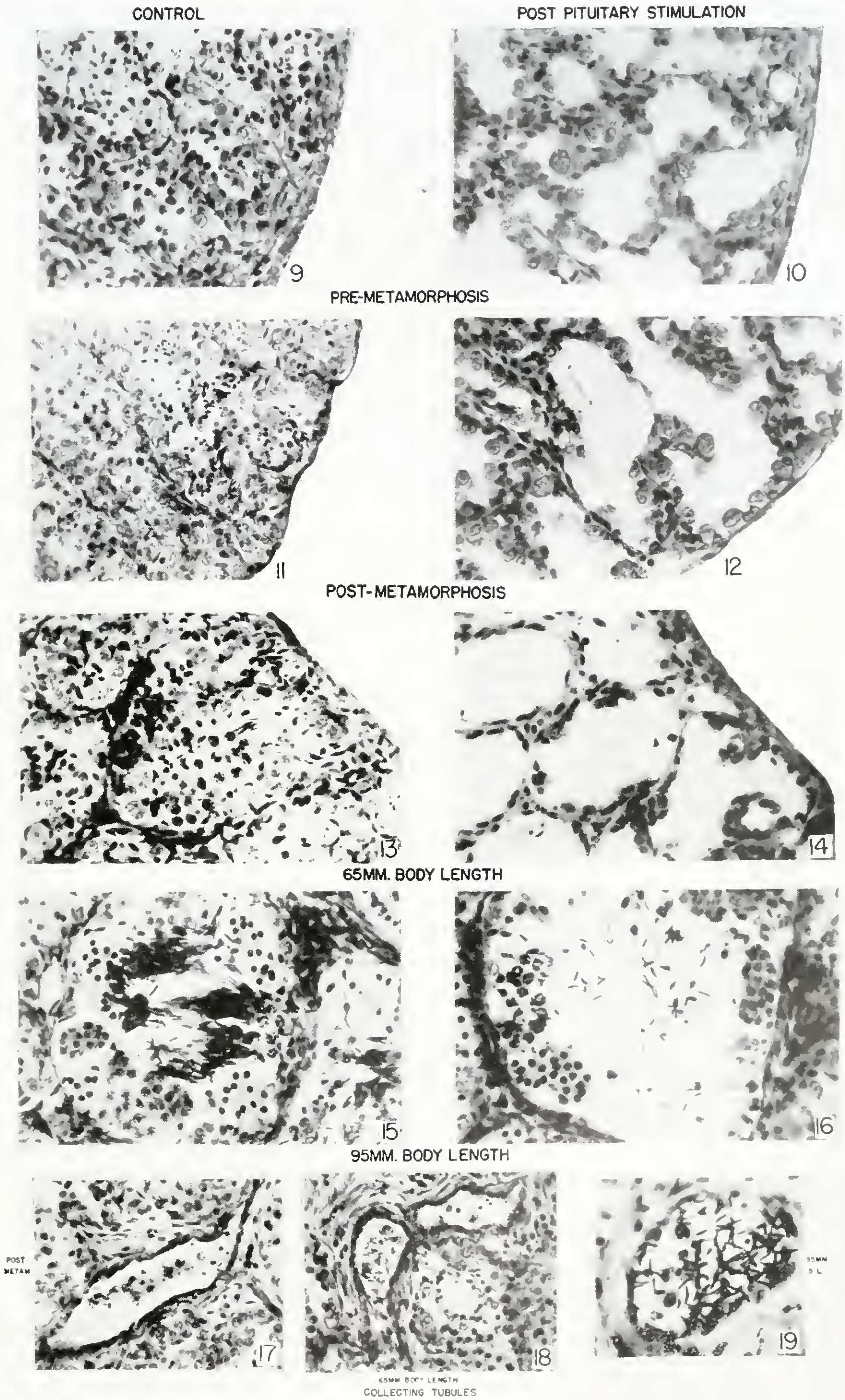

CONTROL
POST PITUITARY STIMULATION
9
10
PRE-METAMORPHOSIS
11
12
POST-METAMORPHOSIS
13
14
65MM. BODY LENGTH
15
16
95MM. BODY LENGTH
POST METAM
17
18
95MM B.L.
19
65MM BODY LENGTH
COLLECTING TUBULES

Plate II. Effect of anterior pituitary hormone on seminiferous and collecting tubules *R. catesbiana.*

tubule there appears to be a compensatory reduction in the amount of interstitial tissue, due, no doubt, to the stretching of this tissue in the general growth of the gonad.

Upon stimulation of this testis by the anterior pituitary hormone of the same species, there is a loss from the gonad of all or most of its freely associated cells and cell groups (Figs. 6 and 14). The gonad seems to have its peripheral tubules emptied the more completely, and, upon closer examination, it is noted that often there remain only the scattered primary spermatocytes and spermatogonia. The primary spermatocytes are fewer in relative number than in the previous stage studied. This is probably due to the fact that some of these cells have been utilized in the production of the maturation nests of cells, some of which were liberated under hormone stimulation. The maturation stages which are missing from the seminiferous tubules are found within the collecting tubules (Fig. 18) and the vasa efferentia leading from the testis to the kidney.

95 mm. body length stage

Except for size, this testis has the histological appearance of the adult gonad. The length is about 5.1 mm. while the diameter is about 2.5 mm. The seminiferous tubules are fully formed and distinct from each other, and the tunica albuginea has become considerably thickened.

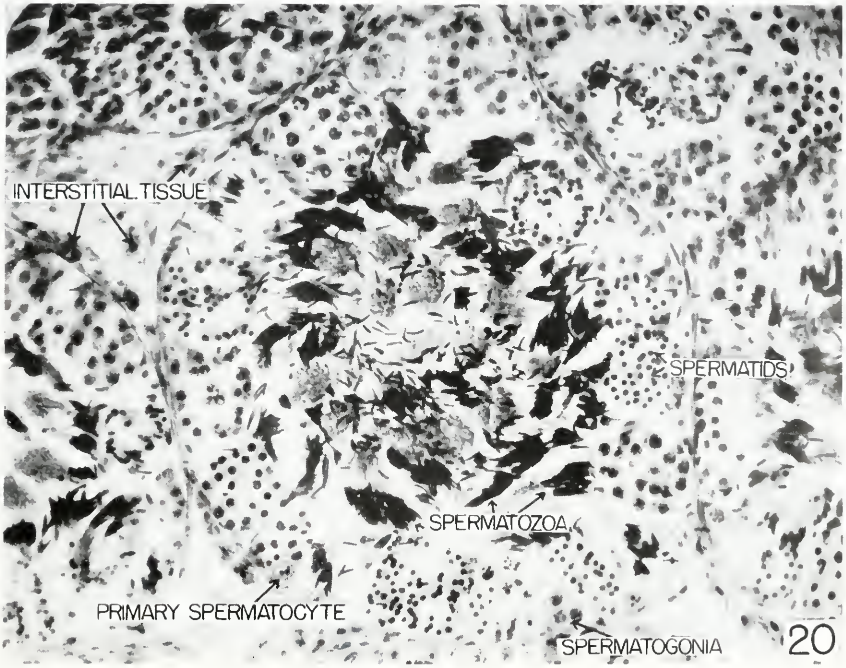
The cysts or nests of cells in the seminiferous tubule include the majority of cells in the more advanced stages of maturation, with abundant fully formed spermatozoa (Figs. 7 and 15). There are many nests of spermatids, and of secondary spermatocytes, but it is with some difficulty that one locates primary spermatocytes.

As a result of anterior pituitary hormone stimulation the lumina of this testis are always enlarged but there is not as complete evacuation of the various cell types as in the previous stage of development studied. That is, some of the secondary spermatocytes and spermatids remain unaffected by the hormone (Figs. 8 and 16), but these same cell types are found within the collecting tubules (Fig. 19) and in the related vasa efferentia.

While the obvious response of the testis as a whole is similar to that of earlier stages of development, there seems to be a greater resistance of many of the cell types, particularly the earlier stages, to evacuation.

The mature bullfrog, body length of more than 120 mm.

The testis of the mature bullfrog measures about 9.8 mm. in length and 5.4 mm. in diameter, being oval in shape and yellowish-white in color. The interstitial tissue is stretched until it remains only as a thin line between seminiferous tubules. Depending upon the season of the year the maturation picture will vary but at the time of this experiment (August) there is normally heightened sexual activity which means abundant mature spermatozoa and abundant reserve cells ready to proceed through maturation, immediately following amplexus and elimination of the mature gametes. Such a picture is seen in Figure 20. It is difficult to locate any spermatogonia or primary spermatocytes, although they are present, due to the abundance of later maturation stages. The mature spermatozoa are in clusters with their heads buried in the cytoplasm of Sertoli cells, and with their tails filling the lumina of the seminiferous tubules. Such a testis is excellent for the study of all of the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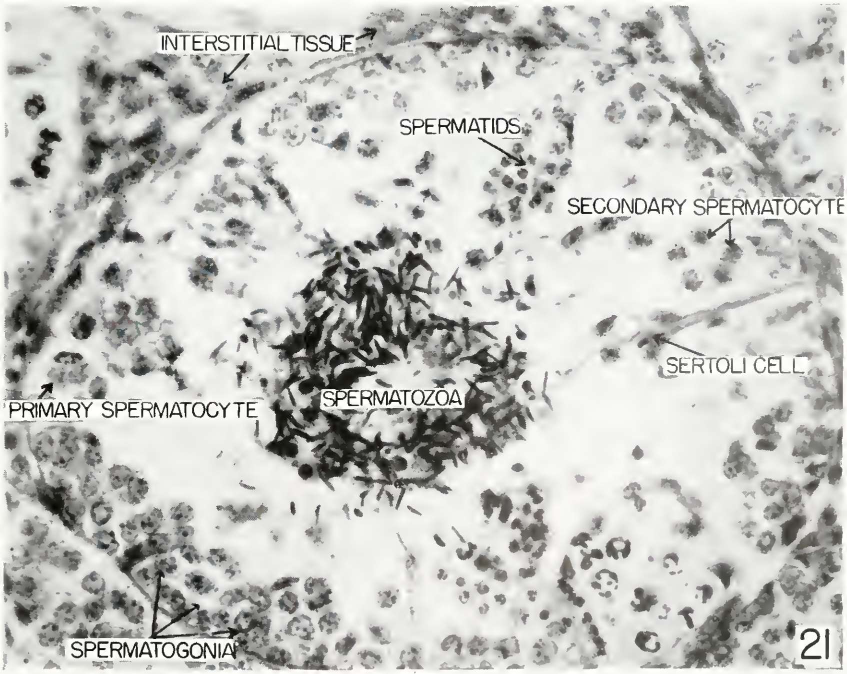
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21

POST PITUITARY STIMULATION

PLATE III. Seminiferous tubules of adult *Rana catesbiana.*

steps in maturation, but, in contrast with the younger frogs there are relatively few of the early maturation stages, i.e., spermatogonia.

Upon stimulation by anterior pituitary hormone the picture becomes somewhat clearer, for the seminiferous tubules are emptied of the mature spermatozoa and an occasional spermatid, and consequently the other stages of maturation within the seminiferous tubules become visible (Fig. 21). The collecting tubules and the related vasa efferentia, however, contain almost exclusively the mature spermatozoa indicating a resistance to liberation of the earlier maturation stages. This resistance was not in evidence with the gonads of younger stages. Sperm release is independent of any overt sex behavior on the part of the male, being a direct response to the anterior pituitary hormone stimulation.

Discussion

The presence of smooth muscle fibers in the ovaries of the frog has been demonstrated (Rugh, 1935) and the contraction of the lobes of the ovary has been seen in highly magnified moving pictures. It has further been demonstrated that the anterior pituitary hormone acts not directly upon the gonad but by way of either or both the nervous and the circulatory systems (Rugh, 1935 and Samartino and Rugh, 1945). This hormone, further, has been shown to stimulate other secondary sexual characters such as the thumb pad of the adult (Glass and Rugh, 1944) and the Mullerian ducts of the immature *Rana pipiens* (Schreiber and Rugh, 1945). In view of these and other findings, it is now clear that upon injection of the anterior pituitary hormone of the frog into the body cavity of the frog, supplementing its own glandular secretions, both the primary and secondary sexual characters are stimulated and the ovary (at least) utilizes its smooth muscle elements to free its follicles of their enclosed eggs.

Puckett (1939) and previously Swingle (1921 and 1926) found a race of bullfrog tadpoles in which there were so-called undifferentiated gonads, particularly in the male, known as protestes. These protestes reacted to daily injections of various mammalian hormones (pituitary and gonadal) by showing precocious development and differentiation, particularly when male sex and pituitary hormones were injected simultaneously. There was no influence on the differentiation of the gonads with regards to sex, but rather the pituitary treatment determined the rate at which the gonads grew. The variety of *Rana catesbiana* used in the experiments reported in this paper did not show the stage of undifferentiated gonads described by Puckett, but they did show a decided response to the amphibian pituitary alone. Adult female frogs have been found to be almost totally resistant to mammalian pituitary extracts (Rugh, 1935 and 1942), while the closely related toads and salamanders would respond readily.

Recently Schreiber and Rugh (1945) treated metamorphosing *Rana pipiens* with the adult frog anterior pituitary hormone and also with the female hormone estradiol benzoate. There hormones were used independently and it was demonstrated that the pituitary hormone acted on the gonad while the estradiol acted upon the gonaducts (oviducts and Mullerian ducts). It is therefore evident that the gonad, whether immature or mature, will respond to anterior pituitary hormone stimulation if the frog is treated with adult amphibian pituitary glands.

The period of this experiment, i.e., 48 hours, was too short to allow demonstration of any growth response of the gonads but there was immediate and drastic response at all stages, whether spermatozoa were present or not, by the elimination of cellular elements from the forming seminiferous tubules. Since there is experimental proof of smooth muscle presence and activity in the frog ovary, and since the post-stimulation picture of the frog testis indicates a greater response at the periphery, it is suggested that here too there may be pituitary stimulation of smooth muscle elements within and surrounding the testis which causes the evacuation of cellular elements. Even if there were evidence of selective action of the pituitary hormone on a specific cell type such an hypothesis would have no real precedent. We are dealing with phases in the maturation of one cell type and, in the immature gonad, all steps in maturation are found within the collecting tubules and the vasa efferentia.

Summary and Conclusions

1. Bullfrog tadpoles and immature male frogs of body lengths of 45, 65, 95, and mature bullfrogs of 120 mm. body lengths, possessing normally differentiating gonads, were treated with anterior pituitary glands from adult frogs of the same species.
2. Scattered spermatozoa normally appear shortly after metamorphosis but by the stage of 65 mm. body length there are relatively abundant structurally mature spermatozoa.
3. The short duration of the experiment, i.e., 72–96 hours, did not allow for any demonstrable growth response of the testis to the anterior pituitary hormone.
4. Even before mature spermatozoa appear, or there is any evidence of a lumen within the seminiferous tubule, the testis of the immature bullfrog reacts to the anuran anterior pituitary hormone by evacuation of cellular elements of the forming seminiferous tubule, and by the formation of exaggerated lumina.
5. Cellular elements, found after pituitary stimulation within the collecting tubules and the vasa efferentia, consist of all stages in maturation except the spermatogonia. Whole cysts of similar cells have been seen, indicating rather violent evacuation of the gonad.
6. The more mature the testis the more resistant are the earlier maturation stages to anterior pituitary hormone release from the gonad, so that in the collecting tubules of the newly metamorphosed bullfrog testis there may be found all stages from spermatocytes to spermatozoa but in the tubules of sexually mature (adult) males there are rarely any cells except spermatozoa.

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INDEX

AMOEBA carolinensis (Wilson, 1900), 8.
Amoebae, fresh water, the contractile vacuole and the adjustment to changing concentration in, 158.
Arbacia punctulata, dilution medium and survival of the spermatozoa of, 177.
Attachment and growth of barnacles, effect of water currents upon, 51.
Attachment and growth of Bugula neritina, toxic effects of copper on, 122.
Autosomal elimination and preferential segregation in the harlequin lobe of certain Discocephalini (Hemiptera), 265.

BARNACLES, effect of water currents upon the attachment and growth of, 51.
BODENSTEIN, DIETRICH. Investigation on the locus of action of DDT in flies (Drosophila), 148.
Brachystethus rubromaculatus Dallas, the elimination of chromosomes in the meiotic divisions of, 19.
Bugula neritina, toxic effects of copper on attachment and growth of, 122.

CARLSON, J. GORDON. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis, 109.
Chironomus salivary gland chromosomes, micrurgical studies on, 71.
Chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas, the elimination of, 19.
Ciliates of the family Ancistrocomidae Chatton and Lwoff, I, 1.
Ciliates of the family Ancistrocomidae Chatton and Lwoff, II, 200.
Contractile vacuole and the adjustment to changing concentration in fresh water amoebae, 158.
Correlation between the possession of a chitinous cuticle and sensitivity to DDT, 97.
COSTELLO, DONALD P. The swimming of Leptosynapta, 93.
CUTKOMP, LAURENCE K. *See* A. GLENN RICHARDS, 97.

D'ANGELO, ETHEL GLANCY. Micrurgical studies of Chironomus salivary gland chromosomes, 71.
DDT, correlation between the possession of a chitinous cuticle and sensitivity to, 97.
DDT, in flies (Drosophila), investigation on the locus of action of, 148.
Dilution medium and survival of the spermatozoa of Arbacia punctulata. II. Effect of the medium on respiration, 177.
Discocephalini (Hemiptera), autosomal elimination and preferential segregation in the harlequin lobe of, 265.

EFFECT of water currents upon attachment and growth of barnacles, 51.
Elimination of chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas, 19.

GRAPHIC method of describing the growth of animals, 141.
Grasshopper neuroblast during mitosis, protoplasmic viscosity changes in different regions of the, 109.

HALL, C. E., M. A. JAKUS, AND F. O. SCHMITT. An investigation of cross striations and myosin filaments in muscle, 32.
HAYASHI, TERU. Dilution medium and survival of spermatozoa of Arbacia punctulata. II. Effect of the medium on respiration, 177.
HENLEY, CATHERINE. The effects of lithium chloride on the fertilized eggs of Nereis limbata, 188.
Heteroagglutinins in body-fluids and seminal fluids of various invertebrates, 213.
HOPKINS, DWIGHT L. The contractile vacuole and the adjustment to changing concentration in fresh water amoebae, 158.

INTRACELLULAR alkaline phosphatases, distribution and properties of, 220.

KOZLOFF, EUGENE N. Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). I. Hypocomina tagularum sp. nov. and Crebricoma gen. nov., 1.
KOZLOFF, EUGENE N. Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmo-

tricha). II. Hypocomides mytili Chatton and Lwoff, Hypocomides botulae sp. nov., Hypocomides parva sp. nov., Hypocomides kelliae sp. nov., and Insignicoma venusta gen. nov., sp. nov., 200.

Krugelis, Edith Judith. Distribution and properties of intracellular alkaline phosphatases, 220.

LEPTOSYNAPTA, swimming of, 93.

Lithium chloride, effects on the fertilized eggs of Nereis limbata, 188.

Locus of action of DDT in flies (Drosophila), 148.

Loosanoff, Victor L. and Charles A. Nomejko. Feeding of oysters in relation to tidal stages and to periods of light and darkness, 244.

MARINE Biological Laboratory Library, serial publications added to the, 88.

Meiotic divisions of Brachystethus rubromaculatus Dallas, elimination of chromosomes in the, 19.

Method of describing the growth of animals, 141.

Miller, Milton A. Toxic effects of copper on attachment and growth of Bugula neritina, 122.

Myosin filaments in muscle, 32.

NEREIS limbata, effects of lithium chloride on the fertilized eggs of, 188.

Nomejko, Charles A. *See* Victor L. Loosanoff, 244.

OYSTERS, feeding of, in relation to tidal stages and to periods of light and darkness, 244.

PHYSIOLOGY of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, Platysamia cecropia, 234.

Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis, 109.

RANA catesbiana, effect of the adult anterior pituitary hormone on the tadpoles and the immature male frogs of, 291.

Richards, A. Glenn and Laurence K. Cutkomp. Correlation between the possession of a chitinous cuticle and sensitivity to DDT, 97.

Rugh, Roberts. The effect of the adult anterior pituitary hormone on the tadpoles and the immature male frogs of the bullfrog, Rana catesbiana, 291.

SALIVARY chromosome manipulation, 71.

Schmitt, F. O. *See* Hall, Jakus, and Schmitt, 32.

Schrader, Franz. Autosomal elimination and preferential segregation in the harlequin lobe of certain Discocephalini (Hemiptera), 265.

Schrader, Franz. The elimination of chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas, 19.

Serial publications added to the Marine Biological Laboratory Library and the Woods Hole Oceanographic Institute Library since February, 1943, 88.

Short, Robert B. Observations on the giant amoeba, Amoeba carolinensis (Wilson, 1900), 8.

Smith F. G. Walton. Effect of water currents upon the attachment and growth of barnacles, 51.

Striations and myosin in muscle, 32.

Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). I. Hypocomina tagularum sp. nov. and Crebricoma gen. nov., 1.

Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). II. Hypocomides mytili Chatton and Lwoff, Hypocomides botulae sp. nov., Hypocomides parva sp. nov., Hypocomides kelliae sp. nov., and Insignicoma venusta gen. nov., sp. nov., 200.

TYLER, Albert. Natural heteroagglutinins in the body-fluids and seminal fluids of various invertebrates, 213.

VISCOSITY changes in grasshopper mitosis, 109.

WALFORD, Lionel A. A new graphic method of describing growth of animals, 141.

Water currents and growth of barnacles, 51.

Williams, Carroll M. Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, Platysamia cecropia, 234.

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CONTENTS

Page

Kozloff, Eugene N.

Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). I. Hypocomina tegularum sp. nov. and Crebricoma gen. nov.... 1

Short, Robert B.

Observations on the giant amoeba, Amoeba carolinensis (Wilson, 1900) 8

Schrader, Franz

The elimination of chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas 19

Hall, C. E., M. A. Jakus, and F. O. Schmitt

An investigation of cross striations and myosin filaments in muscle 32

Smith, F. G. Walton

Effect of water currents upon the attachment and growth of barnacles 51

D'Angelo, Ethel Glancy

Micrurgical studies on Chironomus salivary gland chromosomes 71

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CONTENTS

Page

COSTELLO, DONALD PAUL
The swimming of Leptosynapta 93

RICHARDS, A. GLENN AND LAURENCE K. CUTKOMP
Correlation between the possession of a chitinous cuticle and sensitivity to DDT 97

CARLSON, J. GORDON
Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis 109

MILLER, MILTON A.
Toxic effects of copper on attachment and growth of Bugula neritina 122

WALFORD, LIONEL A.
A new graphic method of describing the growth of animals . . 141

BODENSTEIN, DIETRICH
Investigation on the locus of action of DDT in flies (Drosophila) 148

HOPKINS, DWIGHT L.
The contractile vacuole and the adjustment to changing concentration in fresh water amoebae 158

Volume 90 Number 3

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CONTENTS

Page

HAYASHI, TERU
Dilution medium and survival of the spermatozoa of Arbacia punctulata. II. Effect of the medium on respiration....... 177

HENLEY, CATHERINE
The effects of lithium chloride on the fertilized eggs of Nereis limbata.. 188

KOZLOFF, EUGENE N.
Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). II. Hypocomides mytili Chatton and Lwoff, Hypocomides botulae sp. nov., Hypocomides parva sp. nov., Hypocomides kelliae sp. nov., and Insignicoma venusta gen. nov., sp. nov............ 200

TYLER, ALBERT
Natural heteroagglutinins in the body-fluids and seminal fluids of various invertebrates.......................... 213

KRUGELIS, EDITH JUDITH
Distribution and properties of intracellular alkaline phosphatases 220

WILLIAMS, CARROLL M.
Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, Platysamia cecropia......................... 234

LOOSANOFF, VICTOR L. AND CHARLES A. NOMEJKO
Feeding of oysters in relation to tidal stages and to periods of light and darkness.................................... 244

SCHRADER, FRANZ
Autosomal elimination and preferential segregation in the harlequin lobe of certain Discocephalini (Hemiptera)....... 265

RUGH, ROBERTS
The effect of the adult anterior pituitary hormone on and the immature male frogs of the bullfrog, Rana catesbiana...... 291

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